VASCilia (Vision Analysis StereoCilia): A Napari Plugin for Deep Learning-Based 3D Analysis of Cochlear Hair Cell Stereocilia Bundles

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ABSTRACT

Cochlear hair cells are essential for hearing, and their stereocilia bundles are critical for mechanotransduction. However, analyzing the 3D morphology of these bundles can be challenging due to their complex organization and the presence of other cellular structures in the tissue. To address this, we developed VASCilia (Vision Analysis StereoCilia), a Napari plugin suite that automates the analysis of 3D confocal microscopy datasets of phalloidin-stained cochlear hair cell bundles. VASCilia includes five deep learning-based models that streamline the analysis process, including: (1) Z-Focus Tracker (ZFT) for selecting relevant slices in a 3D image stack; (2) PCPAlignNet (Planar Cell Polarity Alignment Network) for automated orientation of image stacks; (3) a segmentation model for identifying and delineating stereocilia bundles; (4) a tonotopic Position Prediction tool; and (5) a classification tool for identifying hair cell subtypes. In addition, VASCilia provides automated computational tools and measurement capabilities. Using VASCilia, we found that the total actin content of stereocilia bundles (as measured by phalloidin staining) does not necessarily increase with bundle height, which is likely due to differences in stereocilia thickness and number. This novel biological finding demonstrates the power of VASCilia in facilitating detailed quantitative analysis of stereocilia. VASCilia also provides a user-friendly interface that allows researchers to easily navigate and use the tool, with the added capability to reload all their analyses for review or sharing purposes. We believe that VASCilia will be a valuable resource for researchers studying cochlear hair cell development and function, addressing a longstanding need in the hair cell research community for specialized deep learning-based tools capable of high-throughput image quantitation. We have released our code along with a manually annotated dataset that includes approximately 55 3D stacks featuring instance segmentation (https://github.com/ucsdmanorlab/Napari-VASCilia). This dataset comprises a total of 502 inner and 1,703 outer hair cell bundles annotated in 3D. As the first open-source dataset of its kind, we aim to establish a foundational resource for constructing a comprehensive atlas of cochlea hair cell images. Ultimately, this initiative will support the development of foundational models adaptable to various species, markers, and imaging scales to accelerate advances within the hearing research community.

1 Introduction

The last decade has seen a phenomenal advancement in Artificial Intelligence (AI), giving birth to countless architectures, backbones, and optimization techniques that are being continually refined[1,2]. Despite the potential to revolutionize computer vision tasks, many researchers in biological sciences still use computationally primitive and labor-intensive approaches to analyze their imaging and microscopy datasets. One major area where AI-based computer vision tasks could have maximal impact is in pre-clinical auditory research. In our lab, cutting-edge imaging tools and approaches are developed and utilized to gain insights into the structure-function relationship of subcellular structures in sensory hair cells with molecular resolution. These rich imaging datasets present an opportunity to understand how sensory hair cells function in health and disease, including cases of congenital and age-related hearing loss[3–9]. The structural attributes of the cochlea are quite uniform across species. For example, the famously snail-shaped cochlea is governed by a so-called "tonotopic" organization with a gradient of morphological features that change from one end of the cochlea to the other. The result of this organization is that each position

along the length of the cochlea dictates the characteristic frequency of sound to which the tissue responds, therefore directly correlating position with function. In other words, the sensory hair cell organelles called "stereocilia" or "bundles" follow a pattern of increasing lengths as a function of position along the length of the cochlea[10,11]. The cartoon in Fig 1 illustrates how the length of hair cells corresponds to specific frequencies, when the cochlea is unrolled for illustrative purposes. Hair cells are organized tonotopically. Hair cells at the base of the cochlea respond best to high-frequency sounds, while those at the apex respond best to low-frequency sounds. The physical and functional properties of these structures vary systematically along the tonotopic axis of the auditory system. For example, hair bundles might be longer or shorter, or synapses might be more or fewer or have different properties, depending on whether they are associated with high-frequency or low-frequency regions. The association between these attributes and their purpose makes the cochlea an ideal system for investigating the potential of AI in biological image analysis.

In this study, we leverage Napari, an open-source Python tool[12], as the foundation for developing our plugin, chosen for its robust viewer capabilities. The Napari platform has seen a growing number of plugins[13–26] designed to address various biomedical challenges. However, our plugin represents the first such tool tailored specifically for the ear research community, enabling high-precision AI-based 3D instance segmentation and measurement quantification of stereocilia cells.

Previous studies have explored manual segmentation of stereocilia images to quantify measurements for specific research objectives[9,27]. Others have attempted to automate this process using traditional intensity-based methods[28]. However, these traditional approaches typically lack the capabilities provided by AI technologies, which can autonomously extract features and identify regions without relying exclusively on intensity cues. This gap underscores the critical need for advanced AI-driven segmentation techniques that utilize a broader array of features, thereby enhancing both the accuracy and the detail of the segmentation. We identified a few machine learning and deep learning publications. Urata et. al.[29] relies on template matching and Machine learning-based pattern recognition to achieve detection and analysis of hair cell positions across the entire longitudinal axis of the organ of Corti. Buswinka et al.[30] develops a software tool that utilizes AI capabilities; however, this tool is limited to providing bounding box detection and does not offer 3D instance segmentation for the bundles. This significantly hampers the tool's ability to provide shape-level descriptors and accurate measurements. Cortada et al.[31] use stardist deep learning architecture[32] to segment the hair cells, however, they don't provide 3D instance segmentation, they only provide 2D detection based on max projection.

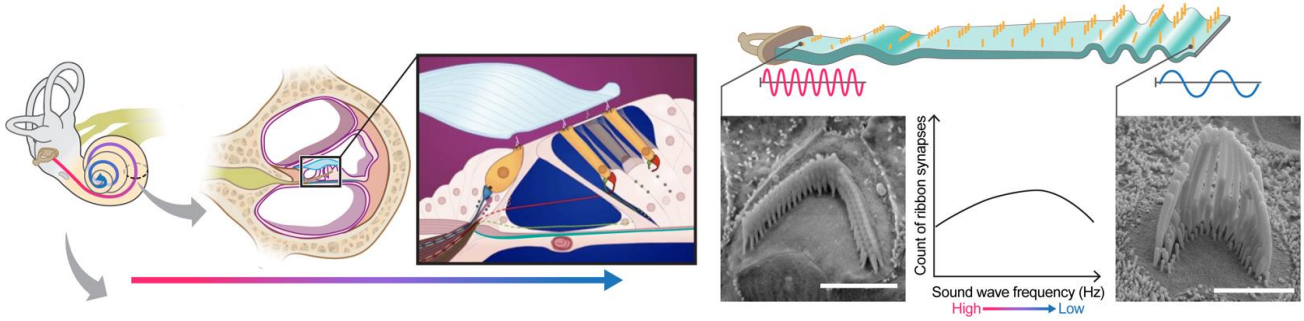


Figure 1. The anatomy of the cochlea[33]. Sensory hair cell stereocilia sit along a tonotopic axis that follows the length of the spiral-shaped cochlea with a striking pattern of increasing stereocilia lengths from the base to the apex of the cochlea. The morphological and spatial features of cochlear hair cell stereocilia follow extremely predictable tonotopic patterns between individuals and species: Stereocilia lengths increase as a function of position along the tonotopic axis of the cochlea, which in turn is reflective of the frequency of sound they are tuned to detect. Thus, the relationship between the morphological and spatial features of these cells and their function are relatively well-defined compared to many other biological systems. Due to their highly patterned organization, cochlear tissues thus present a particularly striking opportunity for automated computer vision tasks.

VASCilia, see Fig 16, equips users with a comprehensive suite of tools for advanced image analysis: 1. Reads and pre-processes 3D image z-stacks. 2. Automatically removes out-of-focus frames from each z-stack. 3. Automatically rotates stacks to align hair cells along the planar cell polarity (PCP) axis. 4. Provides accurate 3D segmentation masks with associated IDs for detailed analysis. 5. Calculates multiple 2D and 3D measurements, including volume, centroid, surface areas, and many others. 6. Automatically determines the tip to base height of each hair cell bundle. 7. Clusters bundles into four rows: IHC, OHC1, OHC2, and OHC3 using K-means, Gaussian mixture models, and deep learning techniques. 8. Computes protein intensities to assess expression levels. 9. Determines bundle orientation with respect to the PCP axis for further analyses. 10. Automatically detects the cochlear region (BASE, MIDDLE, APEX) from which the stack originates using a pre-trained deep

learning model. 11. Allows re-training for other laboratory users to fine-tune models with their own data reflecting potentially different staining protocols and experimental conditions from our training data. 12. Enables the upload and restoration of all analytical data and intermediate steps from a single pickle file, ensuring comprehensive data management and reproducibility. We anticipate that our AI-enhanced plugin will streamline the laborious process of manual quantification of hair cell bundles in 3D image stacks, offering high-throughput analyses with solid interpretation.

Results

1.1 Z-Focus Tracker for 3D Images of Cochlear Hair Cells

In this section, we present the performance and evaluation of our custom network, ZFT-Net, along with comparisons to other commonly used architectures such as ResNet10, ResNet18, DenseNet121, and EfficientNet. Our goal was to classify image frames within each 3D z-stack into three zones: the pre-cellular zone (PCZ), the cellular clarity zone (CCZ) and the noise saturation zone (NSZ) to focus segmentation and analysis on the portion of the z-stack that contains clear cellular structures. The results shown in Table 1 and Fig. 2 highlight the performance of the networks in metrics such as accuracy, precision, recall, specificity, F1 score and error rate, and prediction stability. We found ZFT-Net produced the best results. All metrics were averaged across all three classes and calculated using true positives (TP), false positives (FP), false negatives (FN), and true negatives (TN) derived from the confusion matrix.

One notable feature of ZFT-Net is that it does not produce prediction fluctuation errors, which we named "switches". This consistency in its predictions offers a high degree of confidence, making ZFT-Net our primary choice for VASCilia. As described in 3.3, the preparation of the data and the configuration of CNN played a crucial role in achieving these results. We speculate that ZFT-Net outperforms the other well-known networks because our images are downsampled to 256x256 resolution. Deeper networks tend to lose spatial characteristics in the process, which negatively impacts their performance. The architecture of ZFT-Net, on the other hand, is better suited to preserve these critical spatial features, resulting in better performance across all metrics.

$$\text{Accuracy} = \frac{TP+TN}{TP+TN+FP+FN} \quad \text{Precision} = \frac{TP}{TP+FP} \quad \text{Recall} = \frac{TP}{TP+FN} \quad \text{Specificity} = \frac{TN}{TN+FP}$$

$$\text{F1-Score} = 2 \times \frac{\text{Precision} \times \text{Recall}}{\text{Precision}+\text{Recall}} \quad \text{Error Rate} = \frac{\sum_{i \neq j} \text{Confusion Matrix}_{i,j}}{\sum_{i,j} \text{Confusion Matrix}_{i,j}}$$

Architecture	Accuracy	Precision	Recall	Specificity	F1 Score	Error Rate	Confusion Matrix	Switches
ZFT-Net	**97.66**	**94.14**	**96.01**	**98.48**	**94.97**	**0.03**	$\begin{bmatrix} 237 & 1 & 0 \\ 6 & 72 & 7 \\ 0 & 3 & 159 \end{bmatrix}$	**0**
ResNet18	94.36	89.59	87.46	95.81	88.16	0.08	$\begin{bmatrix} 236 & 5 & 0 \\ 6 & 65 & 23 \\ 1 & 6 & 143 \end{bmatrix}$	9
ResNet10	94.77	92.18	88.65	95.89	89.52	0.07	$\begin{bmatrix} 231 & 3 & 0 \\ 12 & 72 & 22 \\ 0 & 1 & 144 \end{bmatrix}$	6
DenseNet121	95.87	92.43	90.67	97.05	91.21	0.06	$\begin{bmatrix} 241 & 5 & 1 \\ 2 & 69 & 20 \\ 0 & 2 & 145 \end{bmatrix}$	3
EfficientNet	95.46	90.01	89.98	96.68	89.99	0.06	$\begin{bmatrix} 236 & 9 & 0 \\ 7 & 60 & 10 \\ 0 & 7 & 156 \end{bmatrix}$	3

Table 1. Comparison of Architectures Based on Performance Metrics for Z-Focus Tracker. The results highlight that ZFT-Net outperforms all other architectures across all evaluation metrics. Notably, ZFT-Net achieved a switch count of 0, meaning its predictions consistently transitioned from class 0 (pre-cellular zone) to class 1 (cellular clarity phase), and finally to class 2 (noise saturation zone), without fluctuations or misclassifications. This indicates the model's robustness in maintaining a stable and accurate prediction sequence.

1.2 Rotation Correction and Alignment of 3D Image Stacks with PCPAlignNet

The orientation of 3D cochlear image stacks is highly variable due to inconsistencies in imaging parameters, tissue handling, and the complex spiral structure of the cochlea. Automated orientation of cochlear hair cell image stacks can significantly accelerate batch processing, which is essential for efficiently handling large datasets. To address this challenge, we generated training data for PCPAlignNet, which aligns each 3D stack to orient the hair cell bundles along tha planar polarity axis.

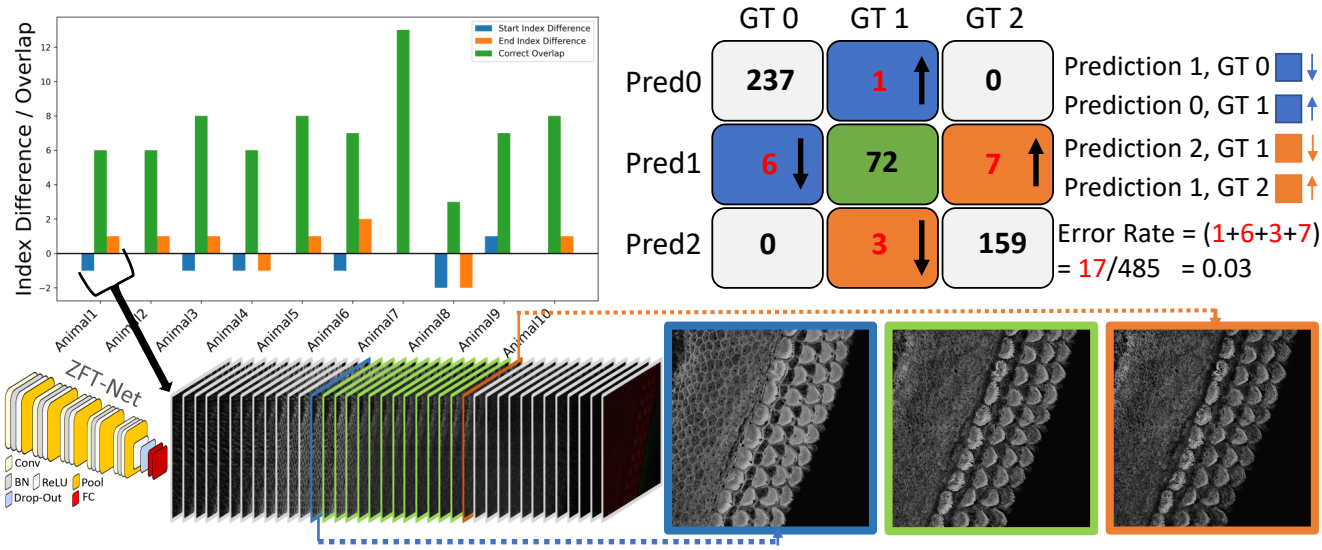


Figure 2. The confusion matrix for ZFT-Net is presented alongside a corresponding bar plot, illustrating the overlapping predictions for the CCZ class, represented by the digit 1. Errors are color-coded and oriented to reflect the different cases in the confusion matrix. GT0, GT1, and GT2 correspond to the Pre-Cellular Zone (PCZ), Cellular Clarity Zone (CCZ), and Noise Saturation Zone (NSZ), respectively. At the bottom of the figure, we provide an example of the first subject's prediction, visualized through a barplot. It shows a smooth transition in prediction from 0 (PCZ), to 1 (CCZ), to 2 (NSZ) without any fluctuation errors, except for two minor boundary errors that are likely due to annotator subjectivity.

After data preparation and augmentation 3.4, our training dataset resulted in 72 folders (classes), each containing 1,831 images. We experimented with several networks, and DenseNet121 yielded the best performance, see Table 2.

During inference, we corrected the orientation of the stereocilia bundles by predicting the rotation angle for each image in the tested stack using the pre-trained model. For each frame, the model produced class scores corresponding to possible angles of rotation. The predicted scores were averaged across all frames in the stack to ensure that the final decision accounted for the model's predictions for every frame. The angle corresponding to the highest average score (class × 5°) was selected as the final predicted orientation. To achieve proper alignment, the images were rotated by 360° minus the predicted angle.

Architecture	MAE	RMSE	Max Error	Median Absolute Error	R-squared
ResNet50	10.53	41.36	180	0	0.86
ResNet18	10	41.33	180	0	0.86
MobileNet	10	41.33	180	0	0.86
DenseNet121	**0**	**0**	**0**	**0**	**1**

Table 2. Performance comparison of different architectures using various error metrics reveals that DenseNet121 has optimal performance across all the metrics.

1.3 A 3D instance segmentation pipeline for stereocilia bundles

3D image stacks of phalloidin-stained cochlea tissues present multiple challenges that make the 3D segmentation of stereocilia bundles a demanding and difficult task. These challenges include non-specific staining, inter-row confluence, intra-row density, noise, intra-cell signal drop-out, inconsistent signal intensity, and tonotopic shape heterogeneity, see Fig 3. Each of these factors significantly complicates the segmentation process and highlights the complexity of accurately identifying and delineating stereocilia bundles, particularly as a 3D instance segmentation task.

Our method utilizes 2D detection on individual frames, which is then followed by reconstructing the 3D object through a multi-object assignment algorithm. For detailed methodology, refer to Sections 3.6.

We partitioned the dataset into training, testing, and validation groups at the stack level to avoid data leakage and mixing frames from different stacks. The training set includes 30 stacks, while the validation contains 5 stacks. Ten stacks are withheld for testing and evaluation. These stacks are categorized into two types: typical and complex cases. Six stacks fall into the typical category, featuring images that are easier to segment due to less overlap among the bundles and well-separated bundle rows. The

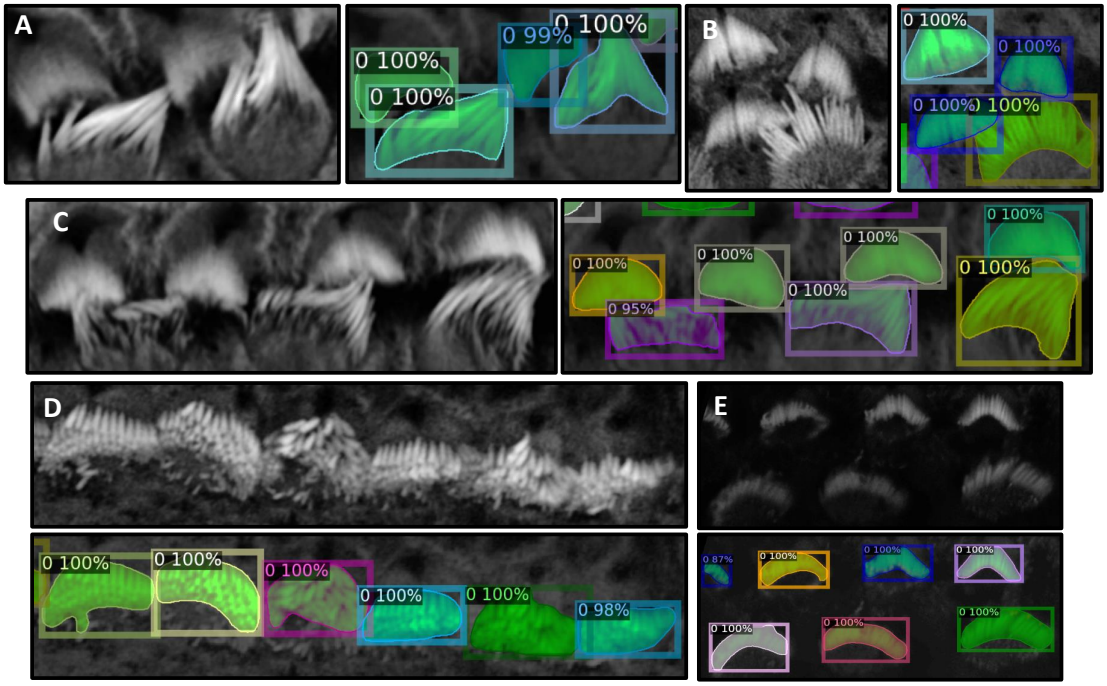


Figure 3. Panels A, B, and C show instances where inter-row stereocilia bundles are in close proximity, yet the algorithm successfully separates them, demonstrating robust performance. **Panel D** presents a scenario where intra-row bundles are tightly clustered; however, the algorithm efficiently distinguishes each bundle, highlighting its ability to resolve complex spatial relationships within the samples. In **Panel E**, despite the raw image being notably dark, the algorithm remains effective in detecting and segmenting individual bundles.

remaining four stacks are classified as complex cases. These contain structures that pose significant challenges for segmentation due to various factors. The objective of this partitioning is to evaluate the decrease in performance when processing challenging cases and check the robustness of the detection algorithm. Figures 3[A-E] illustrate the model's proficiency in accurately detecting the cells and demonstrate the model's effectiveness in overcoming the inherent challenges associated with this task. Fig 4 displays 13 frames from a single dataset or stack that has been segmented in 2D, followed by the application of a multi-object assignment algorithm to reconstruct each 3D object. Details about the algorithm can be found in Section 3.6.

After completing the training process, we employed the trained model to segment each bundle in the images from the testing set. To evaluate performance, we calculate the Intersection over Union (IoU) for 3D volumes by comparing predicted and ground truth segmentation masks. It is defined as $\text{IoU} = \frac{|A \cap B|}{|A \cup B|}$, where $|A \cap B|$ represents the volume of the intersection of the two volumes, and $|A \cup B|$ represents the volume of their union. In addition, we calculate the F1 measure, accuracy, precision, and recall for the predictions against the ground truth. The evaluation process iterates through unique labels found in both ground-truth and predicted volumes, excluding the background label, and computes the IoU for the corresponding labels. This method tracks TP, FP, and FN across varying IoU thresholds. For instance, if one 3D bundle in the ground truth overlaps with two bundles in the predictions, the algorithm considers the one with the largest overlap as a TP and the unmatched one as a false positive. We perform evaluations across a range of IoU thresholds, from 0.1 to 1 in steps of 0.05. In object detection, we do not consider the True Negative (TN) because it refers to correctly labeling background pixels or non-object pixels as such. Since the background or non-object areas can be vast, focusing on TNs would skew the performance metric towards the most common class (background), which does not provide useful information about the model's ability to detect objects of interest.

Figure 5(a) indicates that the average F1-measure and accuracy are 99.4% and 98.8%, respectively, at an IoU of 0.5 for typical test set. In contrast, Figure 5(c) reveals that at the same IoU level, the average F1-measure and Accuracy decrease to 95.7% and 91.9% for complex test set. Maintaining the F1 measure and accuracy above 90% demonstrates that the algorithm effectively handles the significant challenges present in the images. We can observe from Figures 5(b) and (d) that the total number of TPs remains high until the IoU reaches 0.8 and 0.72, respectively. Beyond these thresholds, there is a noticeable increase in both FPs and FNs. When you observe that TPs remain high until IoU thresholds of 0.8 and 0.72, this indicates that the algorithm is quite effective at correctly identifying and matching relevant objects or features in the dataset up to these points. The increasing errors (FPs and FNs) at higher IoU thresholds indicate that the algorithm struggles with precision and

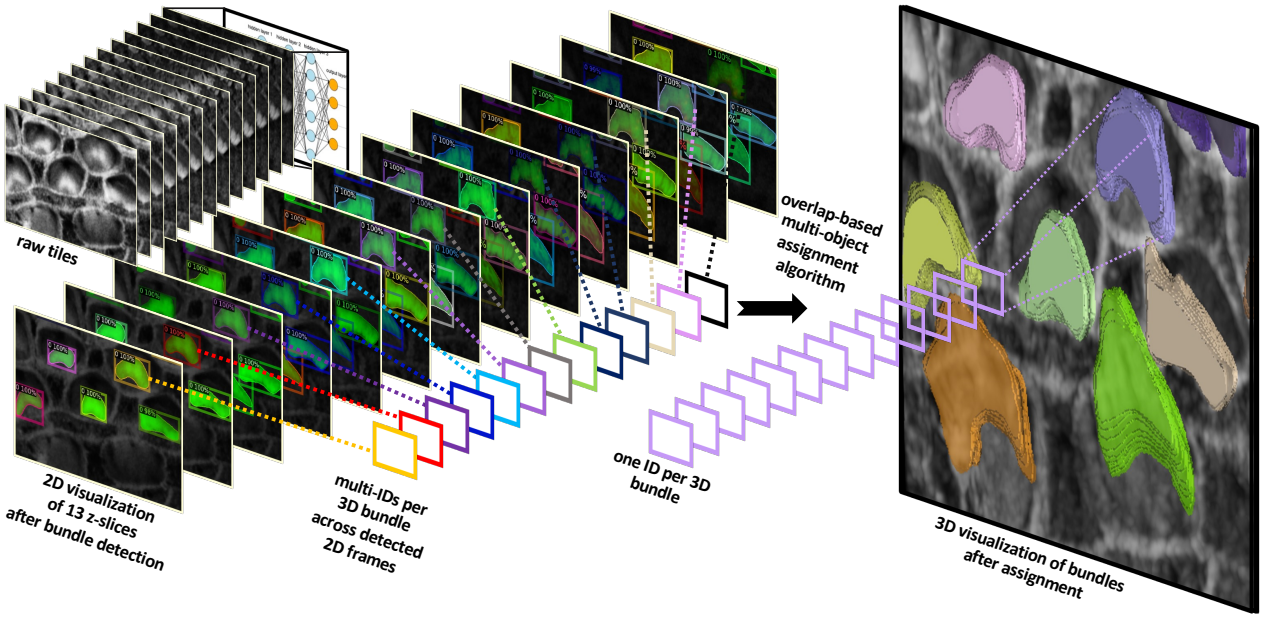


Figure 4. The algorithm successfully detects stereocilia bundles in 2D across successive frames, assigning unique IDs to each detected object within the 2D plane. The variation in IDs across frames reflects the independent detection process in each 2D frame. Subsequently, a multi-object assignment algorithm intervenes to reconcile these IDs, effectively re-assigning them to maintain consistency across frames. This step is crucial for reconstructing accurate 3D objects, ensuring that each bundle retains a consistent ID throughout all frames.

recall balance as criteria become more strict. Figure 6 showcases ten crops of stereocilia bundles: the first row displays the raw crops, the second and third rows features ground truth manual annotations made by two human annotators in the open-source Computer Vision Annotation Tool (CVAT[34]), and the forth row presents our predicted 3D volumes for each bundle. It is evident that most bundles achieve IoU scores between 70 and 80, which visually suggest near-perfect alignment.

The dataset has been annotated by five different human annotators. To put the accuracy of our predictions in a more meaningful context, we investigated the margin of error between annotators on the same stack. We found that the average IoU for 47 instances from a single stack between two annotators is 0.70. Strikingly, the model achieved a higher average IoU of 0.76 with the first annotator and 0.74 with the second annotator. We speculate this is due to the model's ability to effectively average the experience of multiple annotators (five annotators in our case). These results also highlight the difficulty of achieving accuracies beyond a certain amount, as even two human expert annotators only agreed 70% on pixel-based 3D annotations. In addition, Paired t-tests revealed that the model's predictions are significantly better aligned with both Annotator 1 ($t = 3.7399$, $p = 0.0002$) and Annotator 2 ($t = 7.8134$, $p = 0.0000$) compared to the agreement between the two annotators. No significant difference was found between the model's alignment with Annotator 1 and Annotator 2 ($t = -1.7431$, $p = 0.0879$), indicating that the model generalizes equally well across both sets of annotations (Fig. 7). We speculate that the model may be averaging the annotators' varying styles effectively, and that these results underscore the robustness of the model in producing reliable and consistent predictions, which, in some cases, surpass the level of agreement between human expert annotators.

1.4 VASCilia Computational Tools and Measurements

The Napari plugin equips users with essential measurements and deep learning-based tools tailored for analyzing cochlear hair cell stereocilia bundles. Users can accurately obtain up to 15 different 2D and 3D measurements, including volume, surface area, and centroids of segmented regions. Beyond these fundamental measurements, the plugin includes specialized metrics critical for hair cell research, such as calculating stereocilia bundle heights, predicting the region of the cochlea from which the stack is taken (Base, Middle, Apex), classifying hair cells (e.g. inner vs. outer hair cell subtypes), measuring fluorescence signal within each bundle, and determining bundle orientation with respect to the planar polarity axis of the cochlea. These features are designed to support the most common goals of the hair cell imaging research community.

1.4.1 Validation of Stereocilia bundle height measurement

VASCilia significantly reduces the time required for bundle height measurements. The computational approach used for these measurements is detailed in Section 3.7. The plugin provides users with a CSV file that details the height measurements for

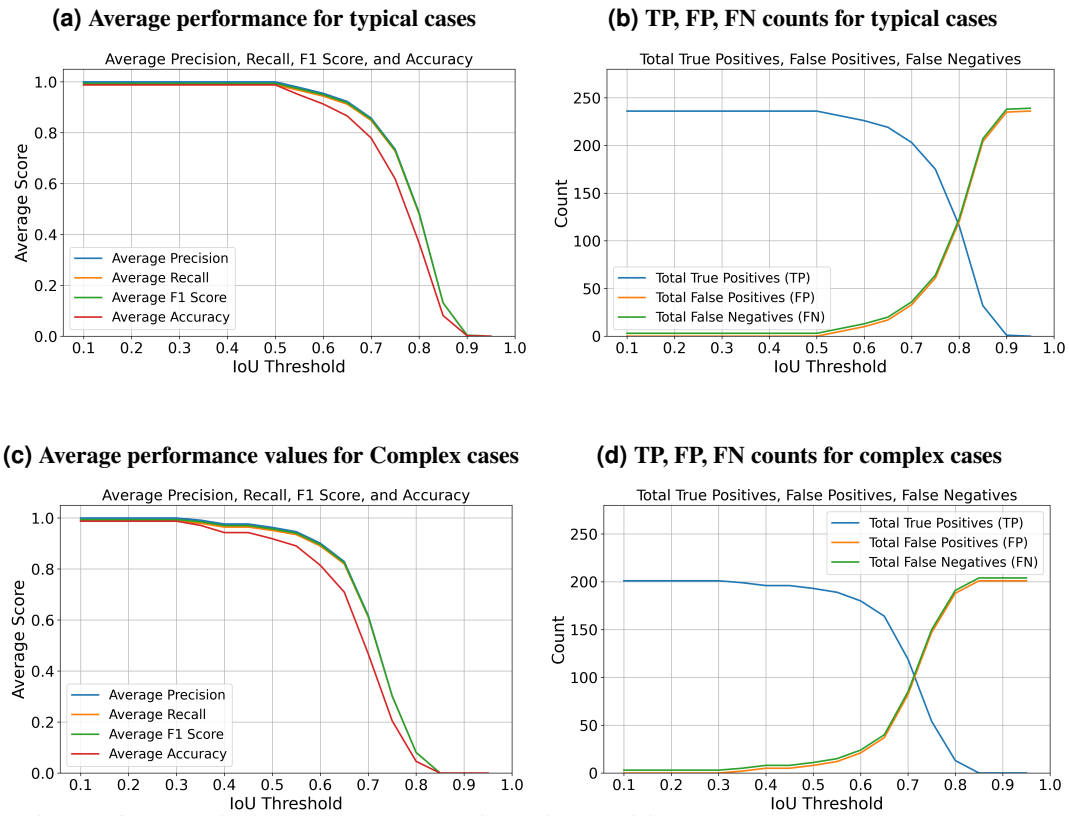


Figure 5. Detailed performance indicators are presented, including Precision, Recall, F1 Score, and Accuracy, along with True Positives (TP), False Positives (FP), and False Negatives (FN). The metrics are evaluated across two categories: typical cases that do not present extreme challenges and complex cases affected by factors such as contrast variations, noise, overlapping bundles, and close proximity between them.

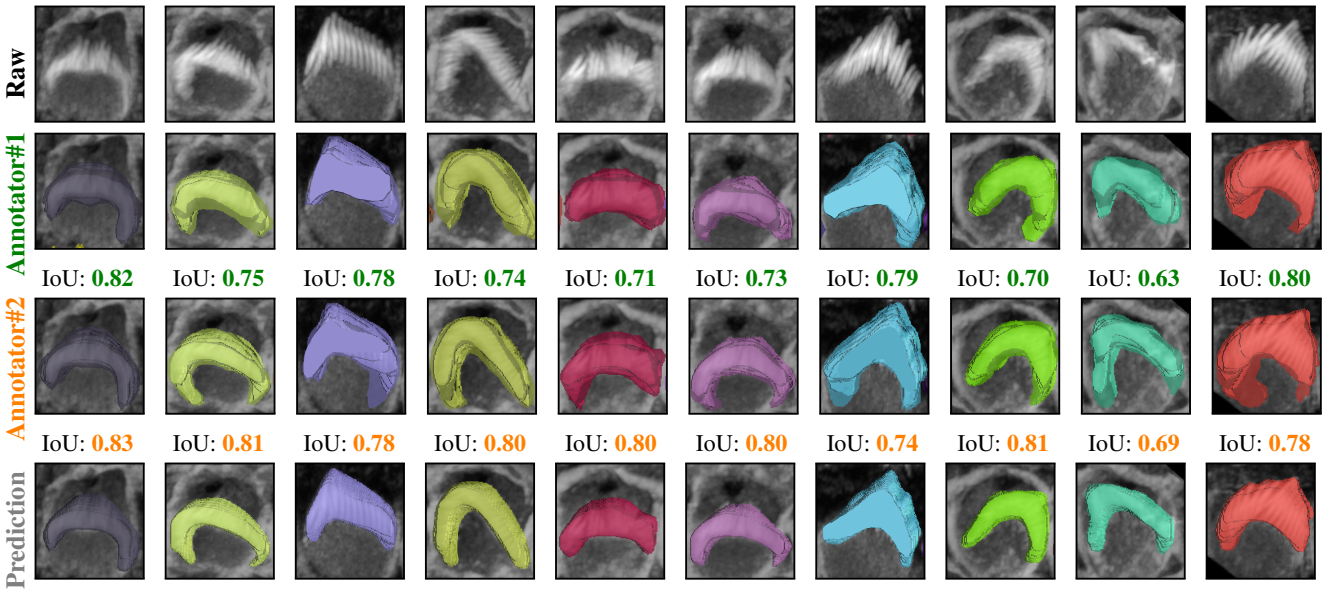
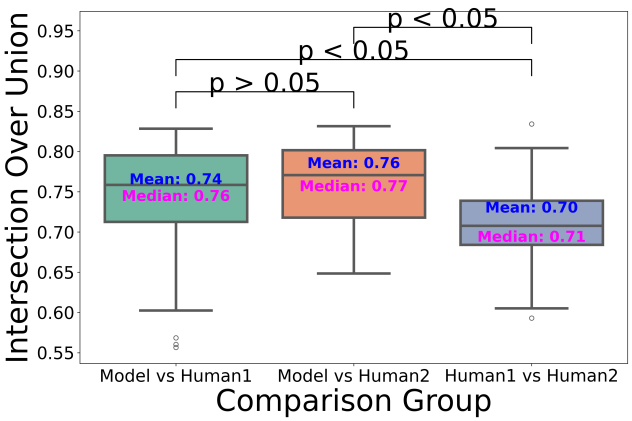
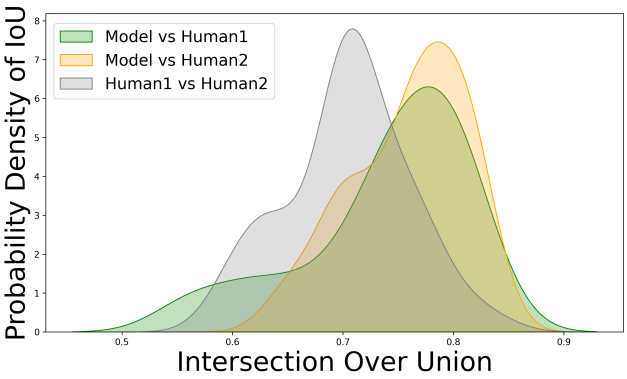


Figure 6. Comparison of raw crops (first row), GT masks for the first human annotator (second row), GT masks for the second human annotator (third row), and the Predicted masks (forth row) for 10 different crops from the same stack, the IoU score underneath the crops indicate the Intersection Over Union score between the 3D GT masks and the 3D predicted masks. We examined the margin of error between two annotators for a single 3D stack. The average overlap was 0.70 between two annotators, 0.74 between one annotator and the prediction, and 0.76 between the second annotator and the prediction over 47 instances across the 3D stack.



(a) Box plot comparing the IoU alignment of model predictions with Human 1 and Human 2, and the agreement between Human 1 and Human 2. Statistical tests indicate that the model predictions are significantly better aligned with both Human 1 ($p = 0.0002$) and Human 2 ($p = 0.0000$) compared to the agreement between the two annotators. Notably, there is no significant difference between the model's alignment with Human 1 and Human 2 ($p = 0.0879$).



(b) A density plot (KDE) estimates the distribution of IoU values by smoothing the data with small curves (kernels) at each point. The y-axis shows the density, representing the relative likelihood of observing the IoU values at different points on the x-axis. The density curves for Model vs. Human 1 and Model vs. Human 2 show considerable overlap, indicating similar IoU distributions between the model and both annotators. In contrast, Human 1 versus Human 2 shows less overlap, suggesting greater variability between the annotators' manual segmentation.

Figure 7. Comparison of the IoU distribution (left) and density plot (right) between model predictions and annotators.

each bundle, each tagged with a corresponding ID. Fig 8 illustrates the computation and Tables 3 and 4 present bundle height validation as measured between VASCilia and two human expert annotators. We found it takes an average of 5.5 minutes to manually annotate each bundle height using the most commonly used open-source microscope image analysis tool, Fiji[35] In contrast, VASCilia significantly reduces this time to just one second to press a button to calculate the height of all the bundles in a single 3D stack (as many as 55), with at most five minutes needed for refining the base and top points of stack bundles if necessary. Thus, measuring the length for 10 stacks, each containing 50 cells, would take 2,750 minutes (approximately 46 hours) with Fiji, compared to only 50 minutes with VASCilia. We conducted both paired t-tests and Pearson correlation analyses to evaluate the agreement between VASCilia and human expert annotators. The Pearson correlation analysis showed very strong positive relationships: 0.942 ($p < 0.001$) between VASCilia and annotator 1, 0.802 ($p < 0.001$) between VASCilia and annotator 2, and 0.830 ($p < 0.001$) between annotators 1 and 2. The paired t-test results indicate no statistically significant differences between the measurements obtained by VASCilia and annotator 1 ($t = 1.868$, $p = 0.078$) or annotator 2 ($t = 1.191$, $p = 0.249$). No significant difference ($t = 0.180$, $p = 0.860$) was observed between the measurements from observers 1 and 2. Together, our findings suggest VASCilia performs comparably to human expert annotators, and that the annotators themselves are consistent in their measurements, see Table 5.

To challenge our approach and demonstrate its utility, we conducted a study involving 18 cochlear stacks from neonatal mice, with samples from three wildtype (WT) animals and three Eps8 KO mice, which have been previously shown to have abnormally short stereocilia[36]. Each animal's cochlea was analyzed across three regions (Base, Middle, and Apex), and within each region, we studied two cell types (IHCs and OHCs). See Fig. 9a for a detailed analysis of stereocilia bundle height variations between WT and KO. The violin plots were generated using data processed through the VASCilia plugin. Consistent with prior studies, VASCilia showed a gradient of increasing bundle heights along the base-mid-apex tonotopic axis for both WT and Eps8 KO mice, with Eps8 KO mice showing significantly shorter bundles than WT. Table 6 presents the statistical summary of IHC and OHC counts in this study.

One limitation about distance computation arises when the entire bundle is bent or when the stereocilia within the bundle are particularly short, such as in the base region of the cochlea in young and/or Eps8 KO mice, where the measured length from tip to bottom may not reflect the tallest stereocilium. To address these issues, the plugin includes event handlers that allow users to manually adjust the highest and lowest points in each bundle. These adjustments are easily made through the intuitive 3D viewer in the Napari interface, enabling researchers to refine measurements as needed.

1.4.2 Fluorescence Intensity Measurements

Understanding signal levels whether they are one or multiple proteins or a particular stain is crucial in various research fields, including cochlear function. The plugin enables the user to get a precise quantification for the signal to allow researchers to

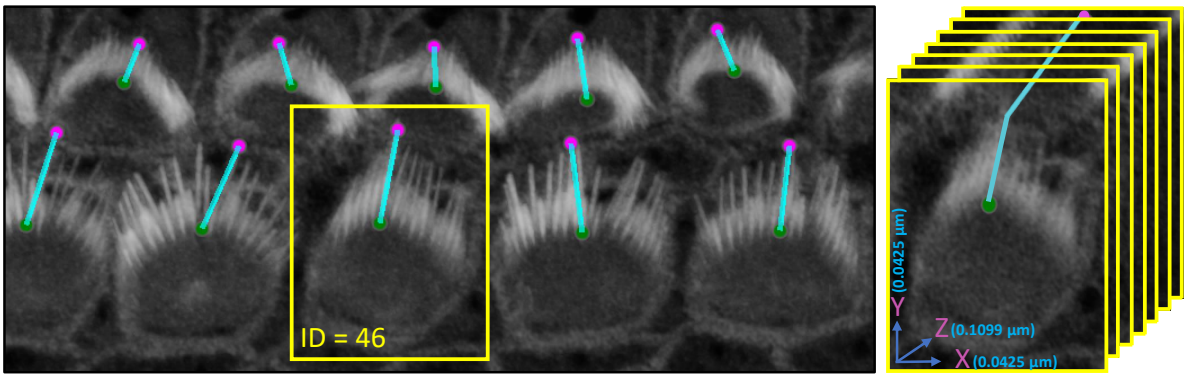


Figure 8. An illustration of the plugin's automated method for measuring the distance from the tip to the bottom of stereocilia bundles. Users can adjust the positions of the upper and lower points. Upon making these adjustments, the plugin's listeners automatically detect the changes, redraw the connecting line, and recalculate the distance accordingly. Left: shows the 3D visualization using Napari. Right: demonstrates how the distance is computed from x, y, and z, considering the physical resolution in microns.

Sample	ID	VASCilia	Observer1	Observer2
DataSet1	2	3.520	3.513	3.431
	19	2.786	2.675	2.883
	25	4.129	3.590	3.947
	32	3.728	3.367	3.613
DataSet2	10	3.816	3.474	3.466
	32	3.028	2.633	2.454
	24	3.748	3.534	3.575
	31	3.572	3.389	3.159
DataSet3	3	2.006	2.189	2.646
	39	1.678	1.897	2.426
	34	2.099	2.202	2.006
	46	4.314	4.543	4.313
	33	3.671	3.624	2.944
	48	3.304	3.519	3.194
DataSet4	3	2.738	2.446	2.916
	47	2.797	2.706	3.206
	17	2.531	2.628	2.044
	54	3.004	2.559	1.965
Statistics		**Mean**	**Median**	**Std. Dev.**
VASCilia		3.137	3.166	0.725
Observer 1		3.027	3.037	0.663
Observer 2		3.010	3.052	0.649

Table 3. Measurements from VASCilia and Observers with Descriptive Statistics

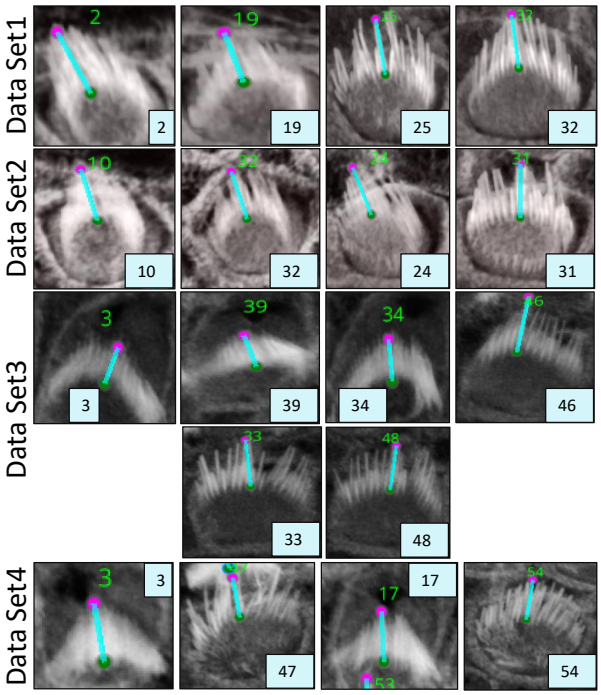


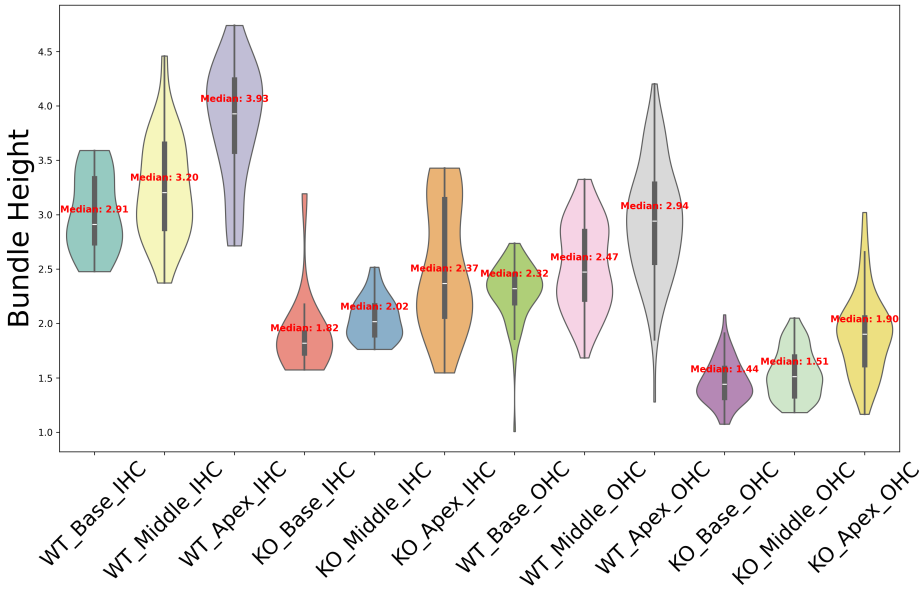
Table 4. Data set crops that are associated to the computations used in Table 3

Comparison	Correlation Coefficient	Correlation p-value	t-test Statistic	t-test p-value
VASCilia vs Observer 1	0.942	< 0.001	1.868	0.078
VASCilia vs Observer 2	0.802	< 0.001	1.191	0.249
Observer 1 vs Observer 2	0.830	< 0.001	0.180	0.860

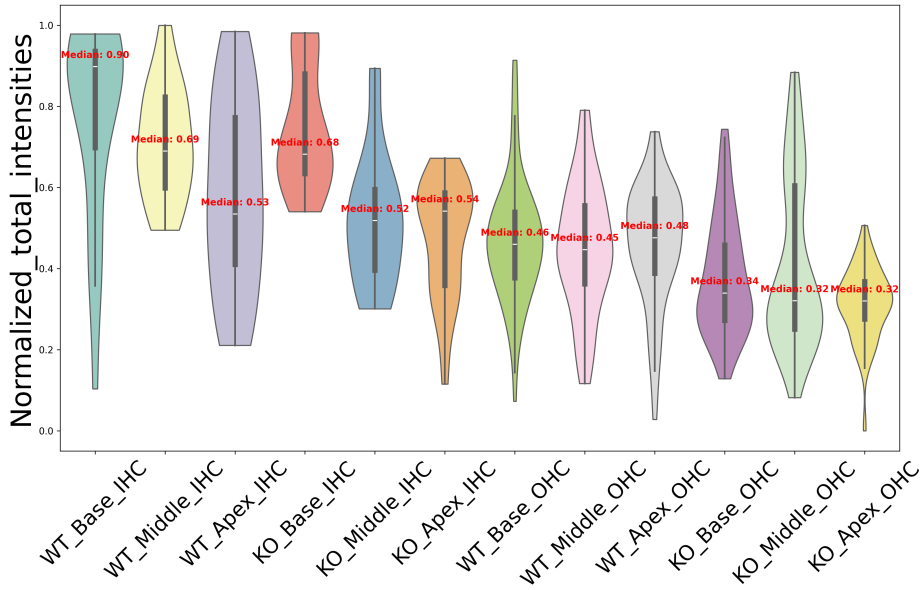
Table 5. Pearson Correlation Coefficients, p-values, and Paired t-test Results

gain deeper insights into its function and potential involvement in hearing loss.

The plugin has a mechanism to calculate both the mean and total fluorescence intensity, subtracting the background intensity to correct for ambient noise. This is achieved by superimposing the 3D segmented masks onto the corresponding fluorescent image slices and aggregating the fluorescent intensities across the z-stack.



(a) The plot demonstrates a clear tonotopic gradient in bundle height, with stereocilia bundles being tallest in the Apex region and shortest in the Base region, particularly for WT groups. KO animals show a marked reduction in bundle height across all regions and cell types, with the most significant reductions observed in Base and Middle regions. The data also suggest that IHCs tend to have taller bundles compared to OHCs.



(b) The plot illustrates the distribution of Phalloidin's min-max normalized total intensities across various groups based on genotype (WT/KO), tonotopic region (Base, Middle, Apex), and cell type (IHC/OHC). IHC_WT groups show the highest normalized intensities in the Base region (median 0.90), with a tonotopic gradient of decreasing intensity from Base to Apex across all groups. KO groups exhibit a significant reduction in intensity compared to WT, with the OHC KO Apex group showing the lowest median intensity of 0.32. Additionally, OHCs generally display lower intensities than IHCs in both WT and KO conditions.

Figure 9. The figure presents violin plots illustrating variations in stereocilia bundle height (top) and Phalloidin intensity (bottom), with the underlying data generated by VASCilia. The study involves P5 data from three mice in both the KO and WT groups, with images acquired from three regions (base, middle, apex) for each mouse. Each image contains one row of IHC cells and three rows of OHC cells, highlighting tonotopic gradients and genotype-related variations across these parameters.

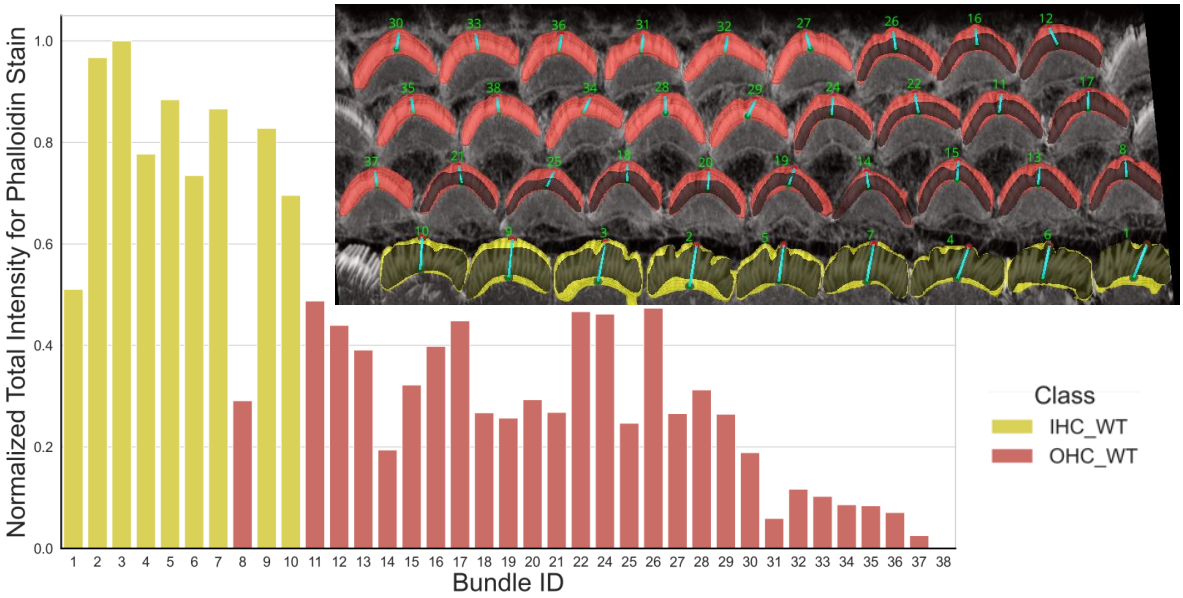


Figure 10. The plot demonstrates the utility and ease with which users can compare data between bar plots and actual cellular images within the plugin. This visual comparison simplifies the interpretation of differences, enhancing the user's ability to analyze variations in signal intensity across different cell types directly within the interface (The Bundle ID is displayed in green within the VASCilia interface). In this example, we explored phalloidin staining and observed that IHCs exhibit higher fluorescence intensity compared to OHCs. This likely reflects differences in the amount of filamentous actin in IHC vs. OHC bundles and may serve as a robust indicator for machine learning models to differentiate between IHCs and OHCs.

The resulting intensities are normalized and plotted, allowing for comparative analysis across different cell types such as inner hair cells (IHCs) and outer hair cells (OHCs) in various categories (OHC1, OHC2, and OHC3). Data for each cell type is stored and visualized through histograms that display the mean and total intensity distributions to provide insights and observation about the protein or signal expressions across the cochlear architecture. To enable further downstream analysis, users will obtain plots for each cell type, resulting in a total of 20 outputs per stack (10 plots and 10 CSV files). See Fig 10 for the total intensity histogram bar for all cells from the Napari plugin, which accumulates the Phalloidin signal in the green channels of the stack. The plugin can also be used to analyze and generate plots for other proteins in different channels as needed by the study.

See Fig. 9b for the results of our challenge case study on the accumulated phalloidin signal between WT and Eps8 KO mice across three regions (Base, Middle, and Apex) for two cell types (IHCs and OHCs). The IHC WT groups show the highest normalized intensities in the Base region (median 0.90), with a tonotopic gradient of decreasing intensity from Base to Apex across all groups. KO groups exhibit a significant reduction in intensity compared to WT, with the OHC KO Apex group showing the lowest median intensity of 0.32. Additionally, OHCs generally display lower intensities than IHCs in both WT and KO conditions. In P5 mice, hair cell bundles in the base of the cochlea exhibited a higher phalloidin signal compared to the middle and apex, which is perhaps surprising given basal hair cell bundles are significantly shorter than apical hair cell bundles. However, these data are consistent with the fact that stereocilia are thinner in OHCs than in IHCs.[37–39], See Supplementary Materials 54 for a visual comparison of stereocilia bundles and associated phalloidin intensity signals between base and apex bundles in WT animals, a pattern observed consistently across all other samples, including KO animals. Table 6 presents the statistical summary of IHC and OHC counts in this study. Overall, these measurements counterintuitively suggest that shorter basal hair cell bundles have higher amounts of filamentous actin than taller apical hair cells, and demonstrate the power and utility of automated, "high-throughput" 3D image analysis facilitated by VASCilia.

1.4.3 Tonotopic Position Prediction (BASE, MIDDLE, APEX)

One of the fascinating features of cochlear hair cell bundles is the so-called "tonotopic" organization, wherein the bundle heights follow a pattern of increasing lengths as a function of position along the length of the cochlea, see Fig 1. Since the frequency of sound detected follows a similar pattern along the length of the cochlea, we were motivated to generate a model that can accurately assess the tonotopic position of the imaged region of the cochlea. To conduct this task, We trained a classification CNN. For more details about the training and architecture, see Section 3.8. This method has demonstrated robust performance,

Table 6. Summary of IHC and OHC counts across different experimental conditions. This study analyzed a total of 136 IHCs and 529 OHCs from 18 3D stacks (9 KO and 9 WT). Each stack included three tonotopic regions: base, middle, and apex.

Tonotopic_Class	KO_Apex	KO_Base	KO_Middle	WT_Apex	WT_Base	WT_Middle	Total
IHC Count	26	15	22	25	23	25	**136**
OHC Count	98	77	88	100	81	85	**529**

achieving a subject-based accuracy of 97%; 28 out of 29 stacks were correctly identified. The single observed misclassification in our testing data involved a stack from the MIDDLE being incorrectly predicted as BASE, which we speculate was due to the close proximity and overlapping characteristics of these two cochlear regions. The covariance matrix for the regions BASE, MIDDLE, and APEX is in Fig 11.

To gain insights into the focus areas of our model during prediction, we employed Gradient-weighted Class Activation Mapping (Grad-CAM), a powerful visualization tool used to explain deep learning-based predictions. Grad-CAM helps visually identify which parts of an image are pivotal for a model's decision-making process. Grad-CAM works by capturing the gradients of the target label, which is the correct class in this scenario, as they propagate back to the final convolutional layer just before the softmax. It then uses these gradients to weigh the convolutional feature maps from this layer, generating a heatmap that visually emphasizes the image regions most critical for predicting the class label. This serves as a sanity check to confirm the model's reliance on accurate features for its predictions. Interestingly, Grad-CAM revealed that the model predominantly focuses on the hair cell bundles to distinguish between the BASE, MIDDLE, or APEX regions. Grad-CAM visualization is shown in Fig 11.

Future directions include exploring the possibility of developing a foundation model capable of accurately predicting cochlear regions across various datasets with different staining and imaging protocols. This effort would undoubtedly require extensive data collection. We believe this work demonstrates that this task is feasible. The Napari-based graphical user interface and interactive visualization tools presented here should help make these methodologies and trained models accessible to the average researcher. We hope these results help motivate collaborative efforts to gather and share more comprehensive imaging data within the inner ear hair cell research community.

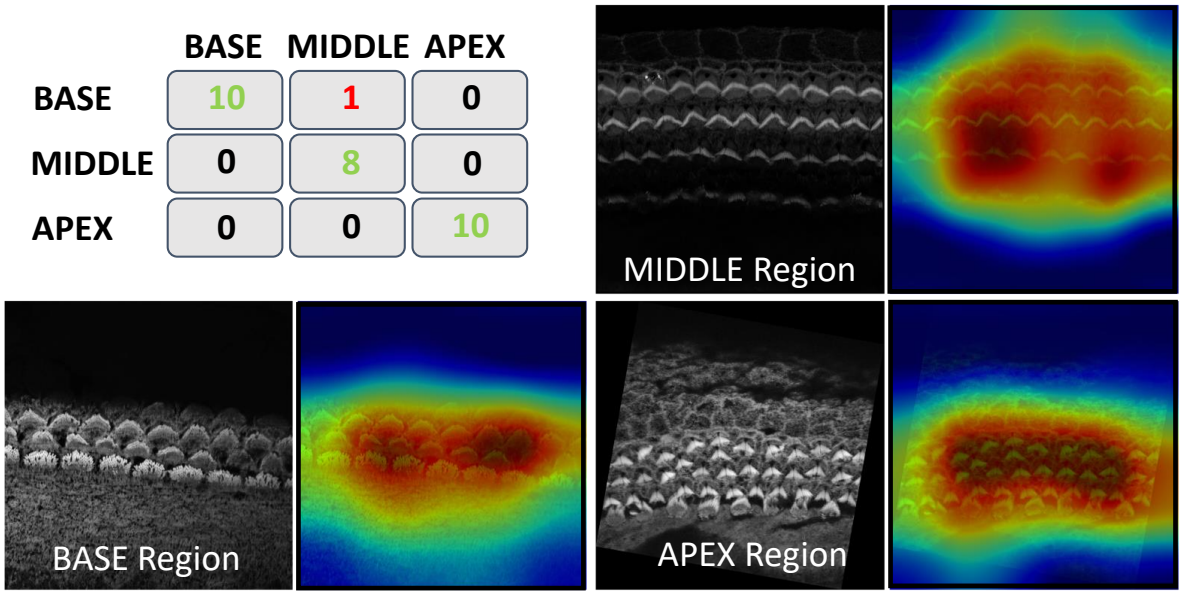


Figure 11. Top left: co-varience matrix for model prediction, others: Grad-CAM Visualization for Base, Middle, and Apex; Resized and Overlaid Response from the Last Convolutional Layer Highlighting Focus on Bundles During Decision Making

1.4.4 High-Throughput Computation of Bundle Orientation

Understanding the precise orientation of stereocilia bundles in the cochlea is critical for deciphering the intricate mechanics of hearing. These hair-like structures are arranged in a "V" shape due to a phenomenon called planar cell polarity (PCP). PCP ensures that neighboring hair cells and their stereocilia bundles are aligned in a specific direction which plays an essential role

in orchestrating the precise orientation of stereocilia bundles, which is fundamental for both the directional sensitivity and efficient mechanotransduction necessary for our sense of hearing. Studying and potentially manipulating PCP holds immense potential for developing better hearing aids, diagnosing hearing loss with greater accuracy, and understanding the mechanisms behind various auditory disorders. In addition, it provides information on the effects of genetic mutations and environmental factors on hearing and facilitates comparative studies that explore evolutionary adaptations in hearing.

The time-consuming nature of the manual PCP measurement methods highlights the need for more automated and efficient techniques to further advance our understanding of this critical process. We identified an automated Fiji plugin, PCP Auto Count, for quantifying planar cell polarity and cell counting[40]. This plugin relies on identifying "chunks" (the hair cell apical surface) and "caves" (the fonticulus) to measure the orientation between their centers. However, this approach was not effective with our images due to the absence of clear caves, especially in low-contrast images. The reliance on distinct caves for orientation measurement poses a limitation for datasets where such features are not consistently visible or distinguishable. For this reason, we developed two automated mechanisms, "Height only" and "Height and Distance", in our plugin to automatically obtain the orientation of the stereocilia bundles based on our 3D segmentation masks:

For the 'Height only' approach, our method pinpoints and records the lowest points on both the left and right sides of the centroid within the 2D projection. Conversely, the 'Height and Distance' method not only locates these points but also measures distances from a specific peak point to identify the most remote and lowest points on each side. While the 'Height only' approach functions perfectly with cells that exhibit a V-shape structure as clear in Fig 12, it is less effective for cells with atypical shapes, commonly encountered in the apex region. This limitation led to the development of the 'Height and Distance' method, which reliably accommodates cells with irregular shapes, see Fig 13.

Following the orientation calculations, the script proceeds to generate lines and angles for each region. It connects the corresponding left and right points and calculates angles using the arctan2 function for deltaY and deltaX, providing a precise angular measurement. These orientation points, lines, and computed angles are then visualized in the Napari viewer through distinct layers, specifically designated for points, lines, and text annotations. This visualization is enhanced with carefully chosen colors and properties to ensure clarity and optimal visibility.

For comprehensive data analysis and record-keeping, a DataFrame containing the IDs and corresponding angles is compiled and exported to a CSV file. To ensure dynamic interactivity within our visualization tool, event listeners are embedded to reflect any adjustments in orientation points directly on the orientation lines and measurements.

1.4.5 Hair Cell Identification (IHC, OHC1, OHC2, OHC3)

The categorization of hair cells into IHC, OHC1, OHC2, and OHC3 in ear biology is not merely a morphological distinction but is deeply tied to their physiological roles, susceptibility to damage, and their integral role in the auditory system. This detailed classification enables more nuanced research exploration, diagnostics, and treatments in audiology and related biomedical fields. Identifying these rows manually is a time-consuming and laborious process. For this reason, we have enhanced our plugin with mechanisms to identify the four rows using three strategies: KMeans, Gaussian Mixture Model (GMM), and deep learning. As a result, this can significantly automate the process, providing high-throughput results for all cells in the current stack in just one second.

1. KMeans: We have implemented KMeans clustering due to its simplicity and efficiency in partitioning data into distinct clusters based on their features. Specifically, we use KMeans to categorize hair cells into four clusters, leveraging attributes such as proximity and density for grouping. This method calculates the centroids of the data points and iteratively minimizes the distance between these centroids and their assigned points, making it highly suitable for fast, general clustering tasks. KMeans proves particularly effective when IHCs and OHCs are distinctly separated and organized in linear rows, especially as it performs optimally without the presence of outliers or significant overlap between rows.

2. Gaussian Mixture Model: The Gaussian Mixture Model is a probabilistic model that assumes all the data points are generated from a mixture of several Gaussian distributions with unknown parameters. By fitting the GMM to the 'y' coordinates of hair cell endpoints, we can predict the cluster for each cell, which aids in categorizing them into distinct groups based on their vertical spatial alignment. This approach is particularly useful in scenarios where the clusters might have different variances, which is often the case in biological tissues. The ability of GMM to accommodate mixed distribution models helps in accurately classifying cells into four predetermined clusters. The Gaussian Mixture Model (GMM) offers flexibility over KMeans by accommodating clusters of varying shapes, sizes, and densities, which is crucial in biological data where such variations are prevalent. Unlike KMeans, which assumes spherical clusters and hard partitions, GMM models the probability of each point's membership in potential clusters, allowing for soft clustering.

3. Deep Learning: While KMeans and GMM provide robust and instant results for many samples, see first row of Fig 14, however, these statistics-based methods can struggle with complex configurations, particularly in samples with outliers, overlapping rows, or non-linear, curvy arrangements. For these challenging scenarios shown in second and third row of Fig 14, we have developed a deep learning approach that utilizes multi-class classification to accurately identify and categorize all hair

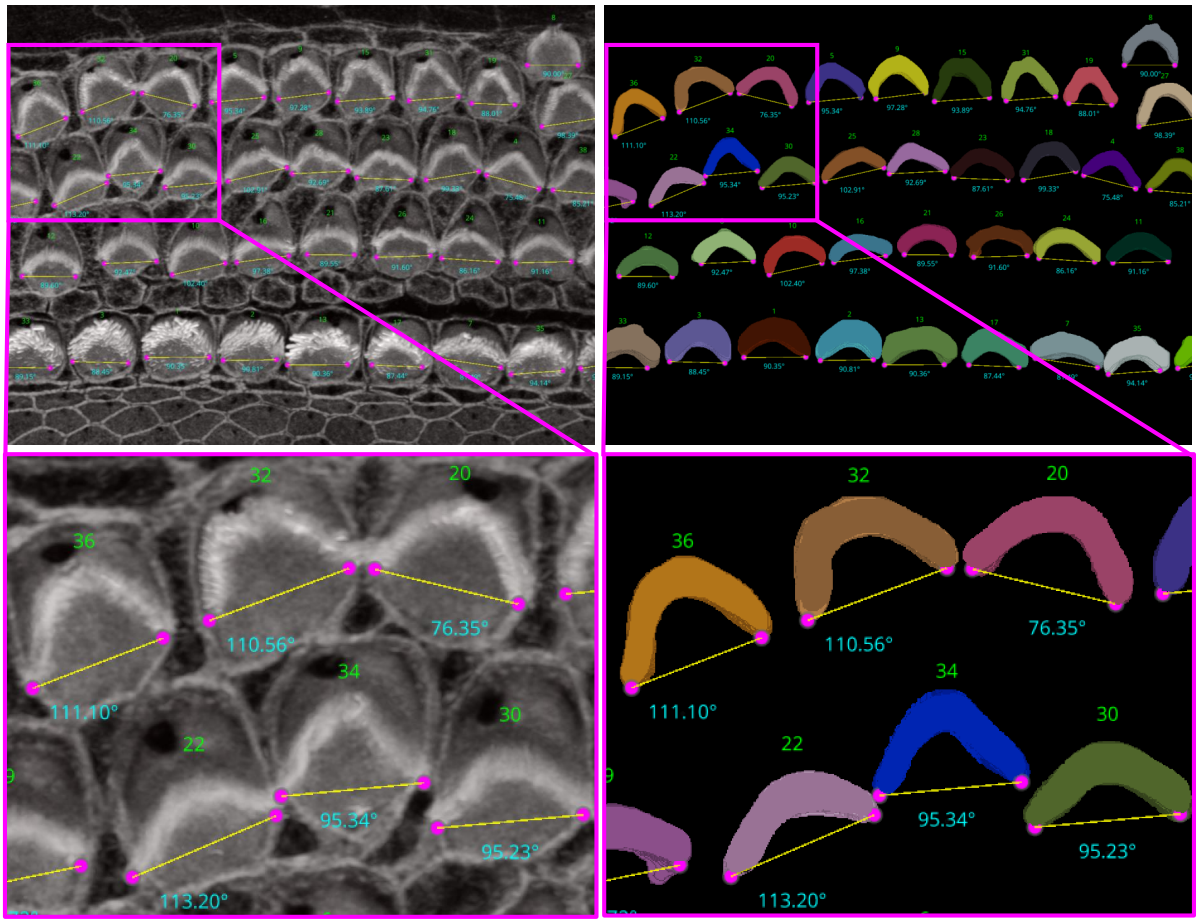


Figure 12. Automated Computation of Stereocilia Bundle Orientation Using a Height-Only Method. Top Left: illustrates the bundle orientations superimposed on the raw data, Top right: displays the 3D segmentation masks with bundle orientation highlighted. Bottom Right and Left are cropped regions for a closer look.

cell bundles, effectively handling the spatial and structural complexities inherent in such data. Check Section 3.9 about the architecture and model training details.

For inference, we focused exclusively on stacks from the Apex region, where the cells frequently display non-linear growth, are closely packed, and often overlap, leading to higher error rates with Kmeans and GMM methods. Conversely, cells in the Base and Middle regions are typically well-aligned. We conducted two experiments: In the first, we tested all Apex data, including 41 datasets from both training and testing phases. In the second experiment, we evaluated 25 stack datasets that were not included in the training set to verify the model's performance on both seen and unseen data.

Figure 15(a) and (b) illustrate the error rates by method and dataset to assess performance per sample. Figures 15(c) and (d) present heatmaps of the errors by method and sample, offering a more visual representation of performance with a color map transitioning from black at the bottom to yellow at the top. These figures highlight the pronounced error frequency in the Kmeans and GMM methods compared to our deep learning approach. Figures 15(e) and (f) display the cumulative errors, providing insight into how errors accumulate linearly per dataset and method. It is notable that the errors with Kmeans and GMM, represented in blue and brown, show a steep increase, whereas the deep learning method demonstrates a more consistent error rate. In Experiment 1, across a total of 1824 cells, the error counts were 202 for Kmeans, 257 for GMM, and 12 for Deep Learning. In contrast, Experiment 2 involved 1102 cells, with error counts of 136 for Kmeans, 169 for GMM, and 11 for Deep Learning. We also conducted a one-way ANOVA (Analysis of Variance) test. This statistical test is used to determine whether there are statistically significant differences between the means of three or more independent (unrelated) groups. We found that there is significant differences between groups with F-value equal to 10.45, A higher F-value indicates a greater degree of variation among the group and P-value equal to 0.0001 which reject the null hypothesis of the ANOVA test. The null hypothesis for an ANOVA test states that all group means are equal.

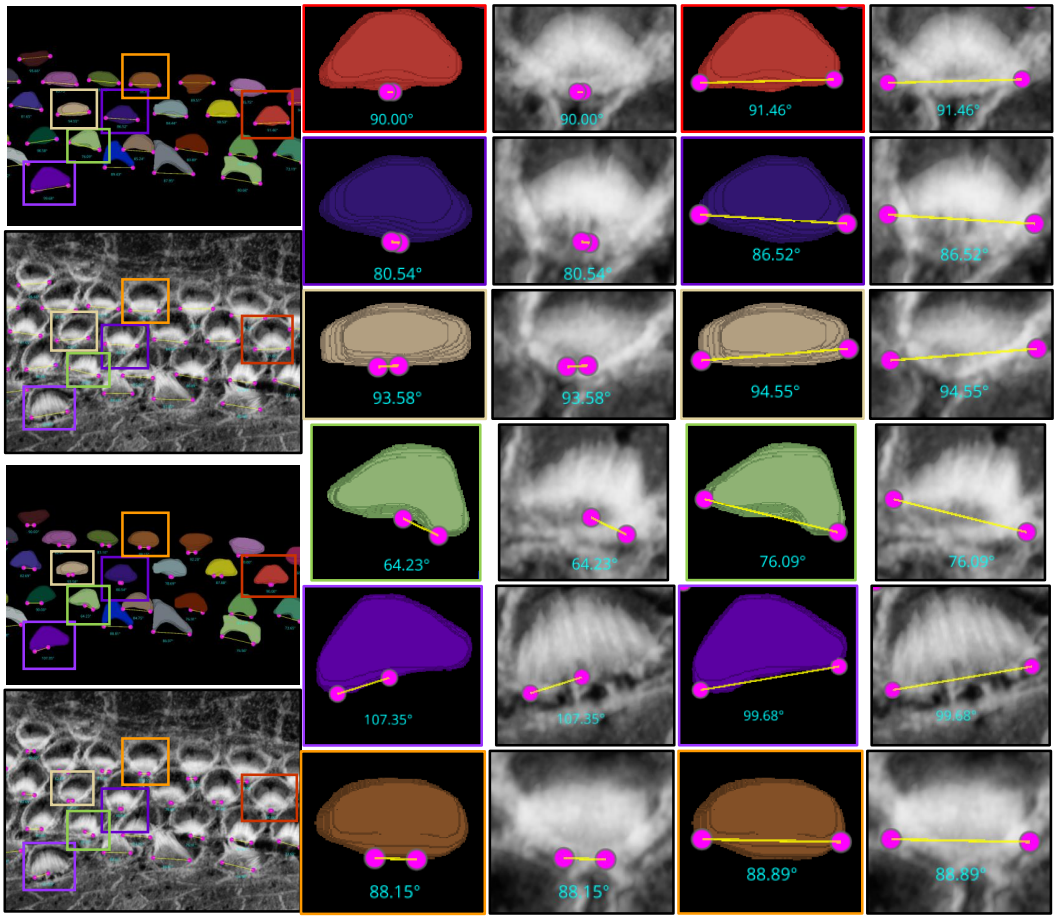


Figure 13. The limitations of the Height-Only orientation computation. While the Height-Only method excels with cells exhibiting a clear V-shape Fig 12, it struggles with some apical region hair cells that lack this distinct structure. To address these challenges, we developed the Height and Distance approach, which effectively handles a wider variety of cell shapes. The visual comparison includes the frames from which the crops are taken (first column), Height-Only results superimposed on the 3D segmented labels (second column) and the original images (third column), alongside the height and distance results superimposed on the 3D segmented labels (fourth column) and the original images (fifth column). We observe that the Height and Distance method overcomes the limitations of the Height-Only computation.

2 Discussion

VASCilia, displayed in Fig. 16, stands out in several unique ways: 1) Purpose-Built for Inner Ear Hair Cell Bundle Research: VASCilia is the first Napari plugin and software analysis tool specifically designed for 3D segmentation of stereocilia bundles. It is equipped with comprehensive analysis capabilities and machine learning models tailored for the inner ear imaging community. 2) User-Friendly Interface: VASCilia features an intuitive, easy-to-install interface that allows users to navigate the tool with minimal effort or expertise. 3) Flexible Application and Fine-Tuning: The flexibility of VASCilia allows users to directly apply it to their own datasets, ranging from mice to humans. Additionally, users can fine-tune their own models using the tool's built-in training module. 4) Batch Processing Capability: VASCilia provides a robust batch processing feature, enabling users to process multiple samples automatically. With automated filtering and rotation of each image stack, all subsequent steps in the pipeline are streamlined. Users simply input the names and paths of their sample files, and VASCilia generates results for each sample, storing them in separate folders with all intermediate results saved for easy access. 5) Review and Collaboration: VASCilia offers a distinctive feature for reloading analysis results from a pickle file, allowing multiple users to review results at a later time, facilitating collaboration and simplifying the review process. For the next three sections, we will provide more details on the VASCilia Workflow and the training section. All workflow steps and buttons are illustrated in the supplementary material. (Figures 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48).

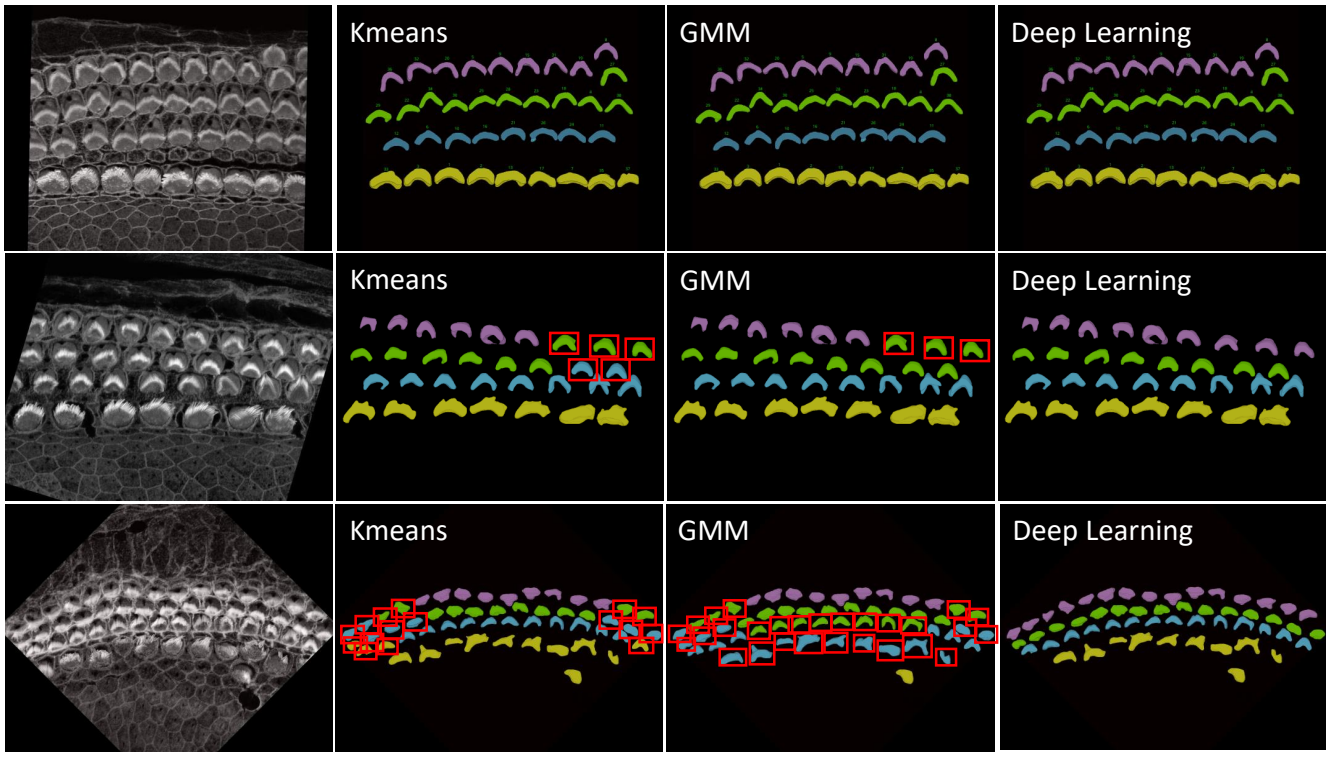


Figure 14. First row: Successful cases for all methods—KMeans, GMM, and Deep Learning—in accurately clustering the four rows into their respective categories are clearly demonstrated. This scenario represents an ideal case where each row is well-separated, linearly aligned, and free from outliers that simplifying the task of accurate clustering. Second and third rows: Failure cases for KMeans and GMM in accurately clustering the four rows into their respective categories are evident: IHC1 in yellow, OHC1 in cyan, OHC2 in green, and OHC3 in magenta. These traditional methods often struggle to precisely segregate the rows due to their inherent limitations in handling complex data distributions, outliers, and overlapping clusters. In contrast, Deep Learning significantly outperforms both KMeans and GMM, providing accurate and reliable clustering for all cell types. Errors are represented by red bounding boxes. For the sample in the second row, there are Five errors in Kmeans, three errors in GMM, and no errors with deep learning. For the sample in the third row, there are Sixteen errors in Kmeans, twenty eight errors in GMM, and no errors with deep learning

2.1 VASCilia Workflow and Features

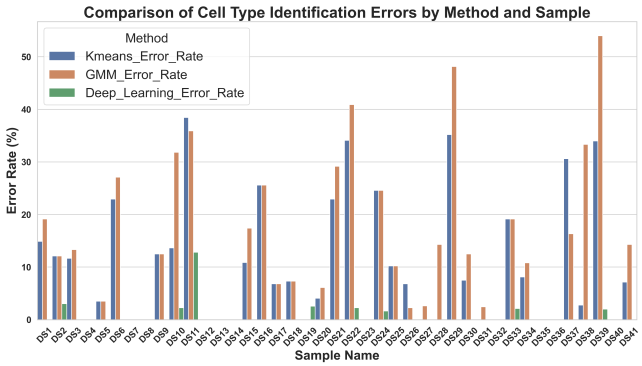
VASCilia begins by initializing all necessary properties for comprehensive analysis, invoking a function called 'initialize_ui' to set up all plugin buttons and prepare the user interface for operation.

Users can either open and preprocess a new dataset using the 'Open Cochlea Datasets and Preprocess' button or upload an analyzed dataset with the 'Upload Processed Stack' button. The plugin supports Zeiss (.czi), Leica (.lif), and Tagged Image (.tif) file formats, with the flexibility to incorporate additional formats. It starts by reading metadata and extracting physical resolution variables for later use in length computations, applies Contrast Limited Adaptive Histogram Equalization for pre-processing, and displays the channels as layers in Napari.

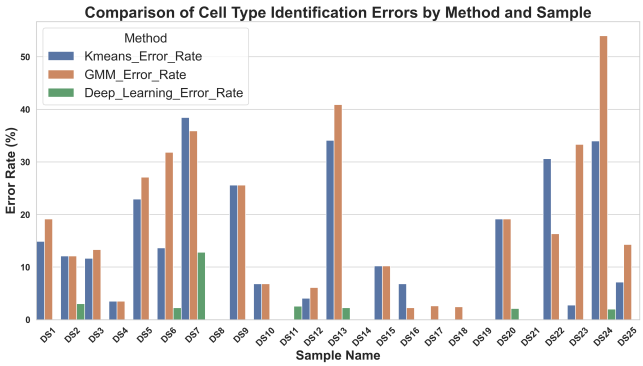
Users initiate their analysis by automatically trimming the stack to isolate the CCZ region of interest, aligning the stack with the tissue's planar polarity axis, and then proceeding with segmentation, reconstruction, and visualization. Subsequently, users can remove unwanted regions, perform measurements, calculate lengths from top to bottom of stereocilia bundles, compute fluorescence Intensity, predict the origin of the stack, determine orientation, and cluster rows into four categories (IHC, OHC1, OHC2, OHC3). All these functionalities depends upon the invoke a function called 'save_attributes,' which efficiently saves all variables used in the analysis in a pickle file. This storage action is implicit, requiring no manual save operations from the user, thus enabling the upload function to retrieve and apply these variables for visualization in the viewer. This setup enables analysts to resume or revisit their analysis at a later time.

After analyzing several datasets, users can compile all generated CSV files related to length computations, orientation, and fluorescence signal responses to analyze and plot observations necessary for their studies.

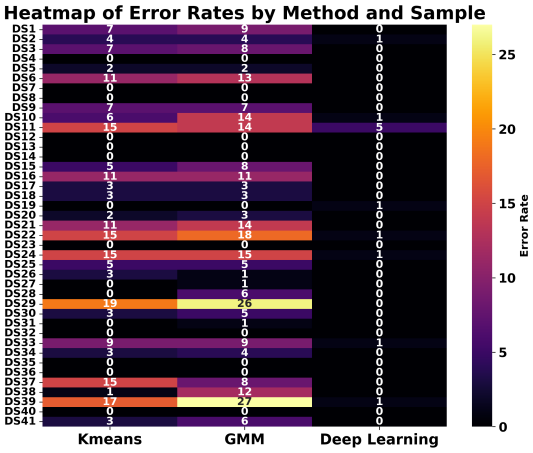
The user can reset Napari for analyzing a new dataset using the 'Reset' button. This feature saves all current variables and



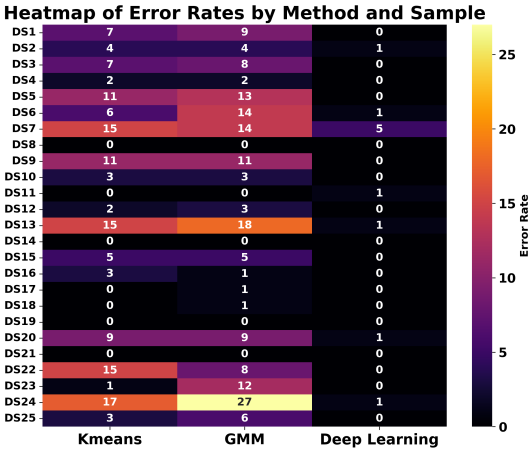
(a) Error Rate by method for all Apex Data



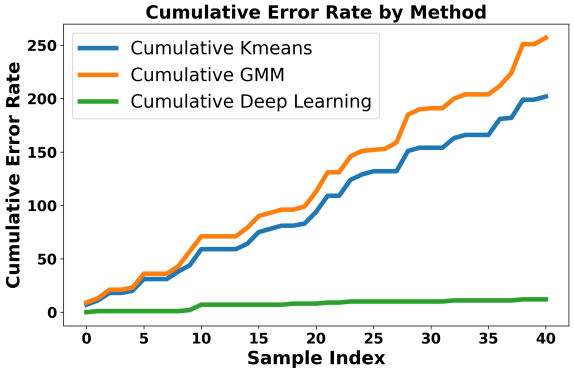
(b) Error Rate by method for all Apex Data for only testing set



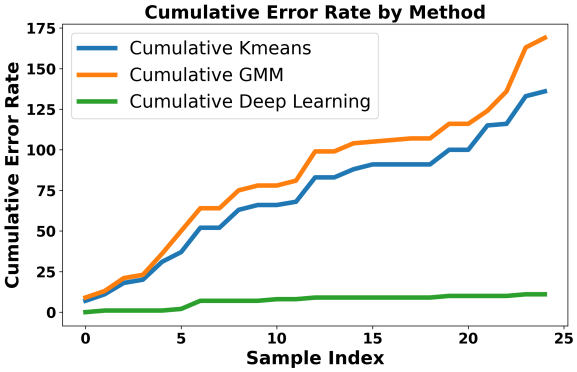
(c) Heatmap by method for all Apex Data



(d) Heatmap by method for all Apex Data for only testing set



(e) Cumulative error by method for all Apex Data



(f) Cumulative error by method for all Apex Data for only testing set

Figure 15. Comprehensive Examination of Error Rates, Heatmaps, and Cumulative Errors for Cell Type Identification in the Apex Region. Subplots (A, B, E, and F) illustrate error rate and cumulative errors, with blue representing KMeans, brown for GMM, and green denoting Deep Learning. Subplots (C and D) utilize the 'inferno' colormap to depict error rates, transitioning from black (low errors) to yellow (high errors), providing a visual gradient of error severity. This color-coded representation aids in distinguishing the methodologies applied across different datasets and highlights the specific error dynamics associated with each method.

then clears them along with all existing layers. This process ensures that the plugin is thoroughly prepared and optimized to handle a new dataset to allow smooth transition between tasks.

2.2 User-Enhanced Accuracy in Automated Measurements

In VASCilia, aside from the segmentation tasks, all automated measurements can be fine-tuned interactively by the user. The plugin is equipped with listeners that actively monitor user interactions related to the adjustment of points, that affect length and orientation computation. This feature ensures that any automated measurements can be further modified to reflect the precise requirements of the user.

Furthermore, for clustering task (cell type identification), VASCilia empowers users to intervene when automated clustering may not align perfectly with the expected outcomes. Users have the flexibility to reassign elements between clusters, correcting any discrepancies. This capability allows for significant refinement of the clustering results to ensure that the automated processe is complemented by user expertise and judgment.

2.3 Training section

VASCilia is fundamentally designed to obtain 3D segmentation of stereocilia bundles, an essential step for all subsequent measurements within the plugin. To ensure adaptability and utility across various labs, VASCilia includes a feature allowing users to fine-tune the existing segmentation model with additional images from their specific datasets. This adaptability is crucial for handling variations in staining techniques, settings, and image dimensions such as height, width, and resolution. This feature is both user-friendly and vital for broadening the plugin's applicability. The training module within VASCilia features seven buttons for ease of use:

Create/Save Ground Truth: allows users to generate a new layer, named 'Ground Truth', within the plugin. This button also enables saving of manually annotated data directly within this layer.

Copy Segmentation Masks to Ground Truth: simplifies the annotation process by transferring existing segmented 3D masks to the Ground Truth layer. This functionality allows users to make precise adjustments to the model's initial predictions rather than starting from scratch to simplify and save the time for the refinement process.

Generate Ground Truth Masks: initiates by identifying and correcting boundary-touching errors, zeroing out pixels where segmented labels overlap. This step is critical to ensure that each pixel retains a unique ID. Furthermore, the function includes a filtering mechanism to manage manually segmented masks sharing identical IDs, maintaining only the largest connected components to ensure each bundle has a distinct ID. This methodical approach is vital for preparing precise ground truth data necessary for effective training processes. Finally, all the masks are saved in a folder pre-defined in the configuration.

Display Stored Ground Masks: allows users to review the stored masks following the automated refinement. This step ensures that all bundles are correctly identified with IDs

Move Ground Truth to Training Folder: automates the transfer of all samples into a pre-configured folder designated for storing training data. This feature is designed to eliminate the need for manual copy-paste operations.

Check Training Data: performs a comprehensive verification of the training data. It ensures that each raw image is paired with a corresponding ground truth and confirms the existence of 'Train' and 'Val' folders within the configured directories, each containing distinct files. Additionally, this function uploads all masks to verify their uniqueness by checking for unique IDs. Should any issues be detected, the plugin will alert the user with a notification of the problem. Conversely, if all checks are passed successfully, the function will display a congratulatory prompt, asking the user to proceed with the training process.

Train New Model for 3DBundle Seg: initiates the training process for the segmentation algorithm. Users have the flexibility to train various models and select the most effective one by simply modifying a path in the configuration file. This plugin is open-source, enabling any user with Python expertise to extend its functionality to suit more specific research needs.

3 Methods

3.1 Description of our 3D microscopy datasets

C57BL/6J mouse P5 and P21 cochleae were harvested and post-fixed overnight at 4°C in 4% PFA. After fixation, cochleae were dissected and tissues were permeabilized in PBS containing 0.3% Triton-X (PBST) for 30 minutes at room temperature. Alexa Fluor 568-conjugated phalloidin was applied in 0.03% PBST containing 3% NGS and incubated for 30 minutes at 23°C. Samples were washed three times with 0.03% PBST for 10 minutes each, mounted in ProLong Glass Antifade (ThermoFisher Scientific, Cat#P36980, Carlsbad, CA, USA) with a #1.5 coverslip, and imaged with a 2.5 μW 561nm laser and a 63x 1.4NA DIC M27 objective on an 880 Airyscan confocal microscope with a 43 x 43nm xy pixel size, 0.110nm z-step size, and a pixel dwell time of 2.3 μs per pixel then processed with default Airyscan processing settings.

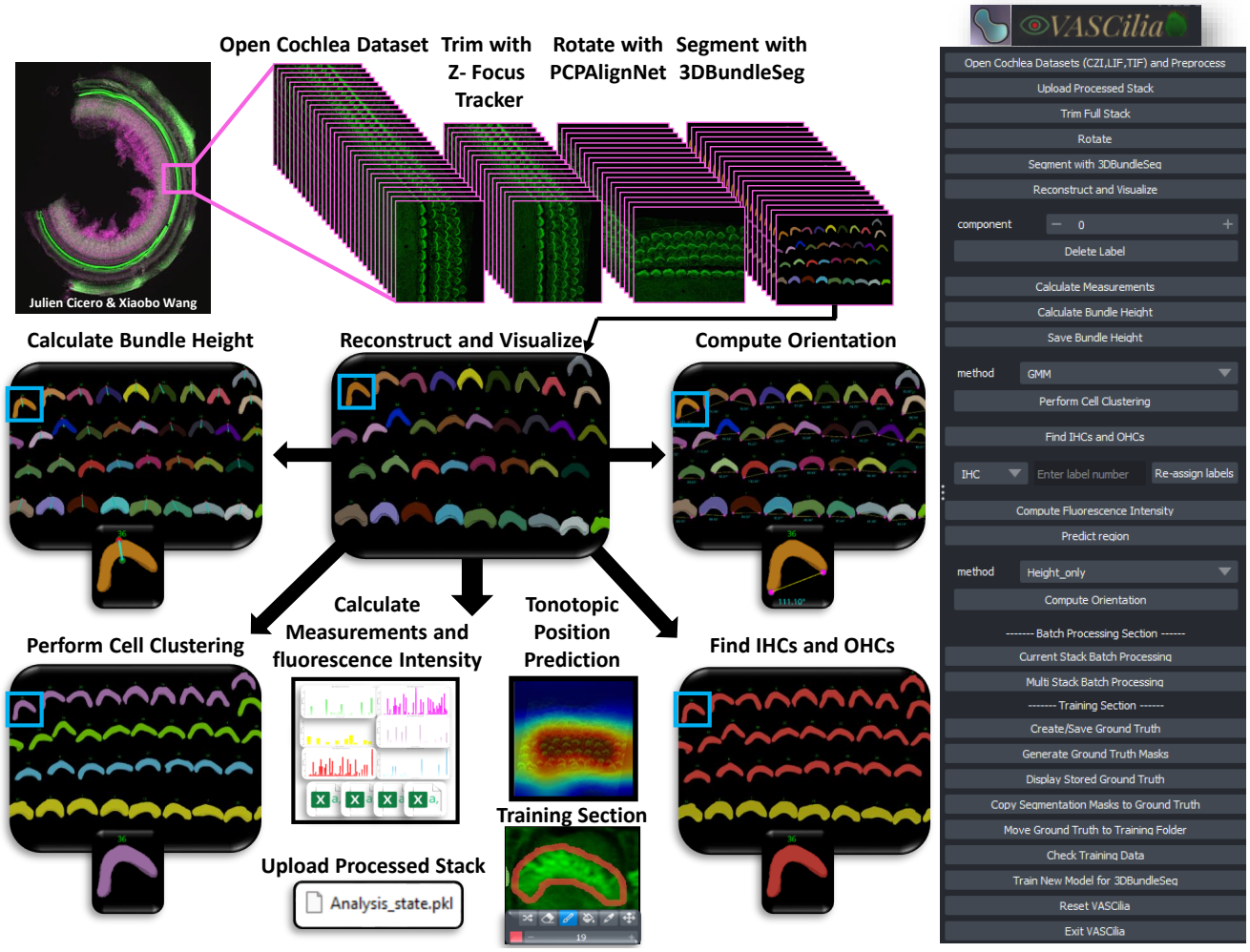


Figure 16. VASCilia enables the ear research community to process their cochlea samples through an end-to-end workflow, all within a user-friendly interface.

3.2 Testing Other Lab Datasets

3.2.1 Artur Indzhykulian's lab datasets

We tested our software without any fine-tuning of the training model on Artur Indzhykulian's datasets[41–43], which included samples with varying mouse ages, illumination conditions, cell counts per sample, image dimensions, and pixel sizes. The software successfully performed 3D segmentation and all subsequent analysis steps on these diverse datasets. See figures 49 and 50 in the supplementary materials.

For this dataset, all procedures were conducted in compliance with ethical regulations approved by the Institutional Animal Care and Use Committee of Mass Eye and Ear, and in agreement with ARRIVE guidelines. Cochleae were dissected in L-15 medium, fixed in 4% formaldehyde (EMS, #15713) in HBSS for 1 hour, and washed with Ca^{2+}, Mg^{2+}-free HBSS. For mice older than P6, fixed cochleae were decalcified in 0.12 M EDTA (pH = 7.0) for 2 days, washed, then micro-dissected, and permeabilized in 0.5% Triton X-100 (Thermo Scientific, #85111) in Ca^{2+}, Mg^{2+}-free HBSS for 30 minutes. Samples were blocked with 10% goat serum (Jackson ImmunoResearch, #005-000-121) in 0.5% Triton X-100 in Ca^{2+}, Mg^{2+}-free HBSS for 1 hour, and incubated in a 1:200 dilution of rabbit anti-PKHD1L1 (Novus Bio #NBP2-13765) overnight at 4°C. Samples were washed with Ca^{2+}, Mg^{2+}-free HBSS and stained for 2 hours in a 1:500 dilution of Goat anti-Rabbit CF568 (Biotium #20099) and phalloidin CF488 (1:20, Biotium #00042) in the blocking solution. Following washes, samples were mounted on slides with the ProLong Diamond Antifade Kit (ThermoFisher Scientific #P36961) and imaged on a Leica SP8 confocal microscope (63x, 1.3 NA objective lens), or an upright Olympus FluoView FV1000 confocal laser scanning microscope (60x, 1.42 NA objective lens).

3.2.2 Liberman's lab datasets

We also tested the software on Liberman's datasets, See Fig 51 and 52 in the supplementary materials. C57BL/6J mice were exposed at 5 weeks of age to noise at 118 dB SPL for 2 hrs, then intravascularly perfused at 8 weeks with 4% PFA. Cochleas were extracted and decalcified in 0.12M EDTA, cryoprotected in 30% sucrose, and stored for 2-3 months at -80°C. After thawing, they were dissected and blocked for one hour at room temperature in PBS with 5% normal horse serum and 0.3% Triton X-100. The tissue was then incubated overnight at 37°C with rabbit anti-ESPN (Sigma #HPA028674 @ 1:100), to label stereocilia, in PBS with 1% normal horse serum and 0.3% Triton X-100. Primary incubation was followed the next day by two sequential 60-minute incubations in an anti-rabbit secondary coupled Alexafluor 647 in PBS with 1% normal horse serum and 0.3% Triton X-100. After immunostaining, pieces were slide-mounted in Vectashield, coverslipped, and imaged on a Leica SP8 confocal with a 63x glycerol-immersion objective (N.A. = 1.3) at 38 nm per pixel in x and y and 250 nm in z.

In addition, Fig. 53 in the supplementary materials shows VASCilia results on human data, despite the fact that no human data stacks were included in our training set. The stacks were imaged on a Leica SP8 confocal using a 63x glycerol immersion objective (Planapo NA 1.3) and the Lightning deconvolution package. The pixel resolution was 0.038 microns in x and y and 0.217 microns in z.

3.3 Z-Focus Tracker for Cochlear Image Data Preparation and Architecture

Our 3D image stacks consist of numerous frames, which we categorize into three distinct zones to better describe the progression of image quality and content throughout the stack:

Pre-Cellular Zone (PCZ): This refers to the early frames where no cellular structures are visible. These frames likely correspond to regions outside the tissue boundaries or the initial imaging volume that has not yet captured the cellular regions.

Cellular Clarity zone (CCZ): This middle portion of the stack contains well-resolved, clearly visible cells, representing the optimal imaging conditions with a high signal-to-noise ratio. Here, the microscope achieves the clearest visualization of stereocilia bundles.

Noise Saturation Zone (NSZ): The later frames where image quality degrades and noise increases significantly, likely due to reduced laser penetration, light scattering, and other optical limitations, leading to fading and distortion of cellular structures.

We define these three zones as distinct classes for training a classifier. The objective is for the classifier to automatically exclude both the **Pre-Cellular Zone (PCZ)** and the **Noise Saturation Zone (NSZ)**, retaining only the **Cellular Clarity Zone (CCZ)**. This approach will significantly reduce processing time while enabling the segmentation model to concentrate on regions containing clear cellular structures.

We have compiled a dataset of 135 3D image stacks of P5 mouse cochlea, using 125 stacks for training and validation, and reserving 10 stacks for testing. Each frame was manually annotated into one of the three defined classes, resulting in 3,355 frames for the PCZ, 1,111 for the NSZ, and 2,088 for the CCZ. After applying data augmentation through rotation, we obtained a balanced dataset with 6,710 frames for each class.

To further enhance the generalizability of the model, we incorporated 13 additional stacks from P21 mice and 22 stacks from Liberman Lab. This expanded the training set to a total of 7,722 frames for each class, improving the model's ability to generalize across different ages, species, and microscopes.

We resized our images to 256 × 256 and tested several networks, including ResNet10, ResNet18, DenseNet121, and EfficientNet. However, our custom network, Z-Focus Tracker Net (ZFT-Net), produced the best results. ZFT-Net consists of five blocks, each containing a convolutional layer, batch normalization, ReLU activation, and a pooling layer. This is followed by an additional ReLU activation and dropout layer, and finally, two fully connected layers.

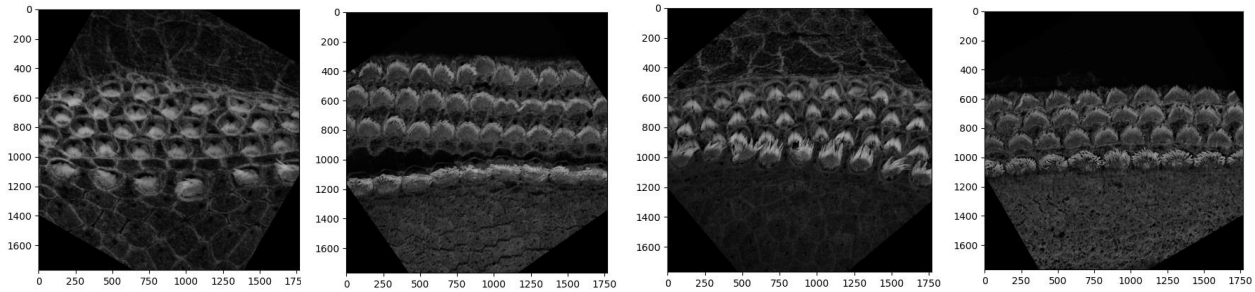
3.4 PCPAlignNet Data Preparation and Architecture

Our P5 and P21 mouse datasets consist of 3D stacks with different orientations ranging from 0 to 360 due to variations in how the cochlear tissue is handled or sliced during the sample preparation process. These small discrepancies in positioning can lead to notable shifts in orientation during imaging. Additionally, the cochlea's spiral and complex three-dimensional structure contributes to the variability in the orientation of the stacks.

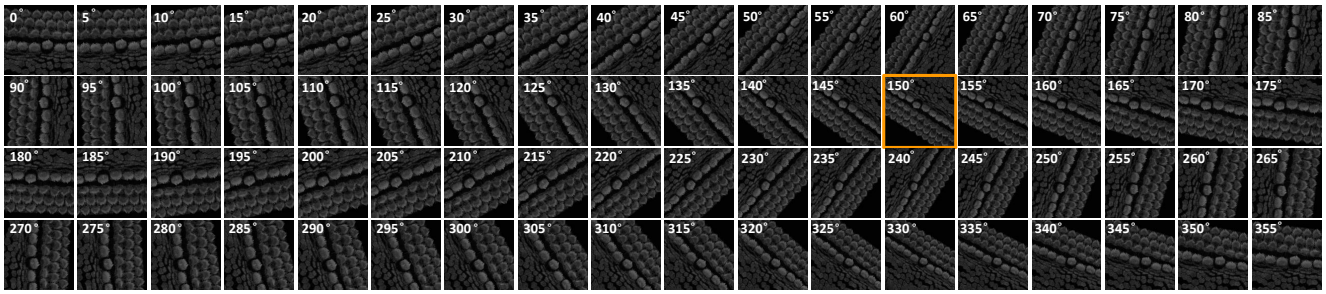
This variability in orientation presents a significant challenge during analysis, as the angle must be corrected so that the stereocilia bundle rows align horizontally with respect to the tissue's planar polarity axis. This is not an easy task because both the images and the bundles exhibit high variability. Even when we manually rotate the images with great precision to appear horizontal to the human eye, some rows may still differ in orientation from others, and bundles can also vary among themselves. As a result, making manual decisions to establish ground truth is extremely challenging. Therefore, we opted to define 72 classes instead of 360. We selected 70% from the stacks of the P5 and P21 data set for training, 20% for validation, leaving 10% for testing. However, these stacks are insufficient to train a robust network capable of predicting across 72 classes, as we do not actually have all the possible angles represented. To address this, we augmented our data set by rotating each frame in every stack to all 72 possible classes (i.e. ranging from 0 to 355 degrees, with 5-degree increments). For training, we experimented with several models, including ResNet50, ResNet18, MobileNet, and DenseNet121. We trained each model for

50 epochs, utilizing early stopping with a patience of 3. The Adam optimizer, along with a cross-entropy loss function, was employed to optimize the model.

A key challenge that caught our attention was the need to avoid the incorporation of empty pixel values in padded regions during image rotation. To address this, we applied a rotation correction in Python, followed by cropping the largest area that excluded the empty regions. Since each frame begins at a different orientation prior to augmentation, the corrected images, after rotation and removal of empty regions, vary in scale. These variations are often beneficial for CNN training, as they improve the network's ability to generalize across different spatial representations of data. See Figure 17



(a) Determining the precise orientation of the frame is challenging, as not all stereocilia bundle rows share the same horizontal alignment. Achieving an exact degree out of 360 is practically impossible, even manually. To address this, we decided to use 72-degree intervals with 5-degree increments. We have adapted the augmentation process, shown in 17b, to increase the number of image samples.



(b) To expand our dataset and represent all possible angles, we augmented each frame by rotating it and cropping the largest area without empty pixels from padded regions. This process results in frames with varying perspectives and scales. In the example shown, the original slide is highlighted in **orange**, while all others are augmented versions.

Figure 17. The top subfigure shows alignment challenges, while the bottom displays the original frame and its augmented versions after rotation and cropping.

3.5 3D Manual Annotation and Dataset Partitioning for Segmentation Task

Training a 3D supervised model that efficiently segments each stereocilia bundle requires manual 3D annotation for many bundles, a process that is both cumbersome and slow. We utilized the Computer Vision Annotation Tool (CVAT)[34] to annotate our 3D samples. CVAT facilitates the drawing of manual polygons with an effective tracking feature, which annotators can use to accelerate the annotation process. We manually annotated 45 stacks using the CVAT cloud application[34], assigning each 3D bundle a unique ID for precise identification. The annotated data were thoroughly inspected and refined by both the author and the biologists responsible for imaging the data.

To maintain the integrity of the data split, we divided the dataset into training, testing, and validation sets at the stack level, thus preventing the mingling of frames from different stacks during partitioning. The training set comprises 30 stacks, the validation set includes 5 stacks, and the testing set consists of 10 stacks, which are further classified into 6 typical complexity cases and 4 complex cases. The details on how many training, validation, and testing 3D instances exist in this dataset can be found in Table 7.

3.6 3D segmentation of stereocilia bundles using 2D detection and multi-object assignment algorithm

Our approach to the 3D segmentation task involves applying 2D detection to each frame, followed by the reconstruction of the 3D object using a multi-object assignment algorithm. We employ the Detectron2 library from Facebook Research[44], using the Mask R-CNN architecture[45] combined with a ResNet50 backbone[46] and a Feature Pyramid Network[47]. This setup leverages

Data Set	IHC	OHC	Total
Training (30 stacks)	270	940	1210
Validation (5 stacks)	47	170	217
Inference (10 stacks)	93	350	443
Total (45 stacks)	410	1460	1870

Table 7. Summary of 3D stereocilia bundle instances across different data sets.

transfer learning from a model trained on the COCO dataset[48]. The algorithm is executed over 50,000 iterations with a learning rate of 0.00025. We focus on a single class, specifically the stereocilia bundle, with a head threshold score set at 0.5.

After getting all the 2D frame segmentation masks across all stacks, the multi-object assignment algorithm involve these steps, see Fig 4:

1. **Initialization:**
   - Set `frame_count` to zero, marking the start of the frame sequence.
   - Create an empty list `tracks` to maintain records of active object tracks.
   - Load the initial frame of the stack to start the tracking process.
2. **Processing the First Frame:**
   - Detect all objects within the first frame and assign each a unique track ID.
   - Store the position and ID of each detected object in `tracks`.
   - Increment `frame_count`.
3. **Tracking in Subsequent Frames:**
   - Load the next frame to continue tracking.
   - Detect all visible objects in the current frame.
   - For each detected object:
     - Calculate the overlap area with each object in `tracks`.
     - Determine the appropriate track based on overlap:
       * If no significant overlap is found, initialize a new track.
       * If an overlap exists, assign the object to the track with the largest overlap and update the track's position.
     - Handle ambiguous cases:
       * If multiple objects overlap significantly with a single track, choose the object with the largest overlap for the track.
       * Consider initiating new tracks for other overlapping objects.
   - Increment `frame_count`.
4. **Loop Through Remaining Frames:**
   - Repeat the process in Step 3 for each new frame until the end of the stack sequence.
5. **Finalization:**
   - Assemble and output all completed tracks for further analysis.
   - Conclude the tracking algorithm. At this stage, each cell or bundle is assigned a unique ID based on the tracks to enable the user to visualize them in Napari.

3.7 Stereocilia bundle height measurement

Hair cell stereocilia bundles are essential for hearing, as they convert acoustic vibrations into electrical signals that the brain detects as sound. Many deafness mutations cause bundle defects, including improper elongation. Accurately and consistently measuring stereocilia bundle heights in 3D images is therefore critical, but unfortunately laborious and costly, in particular for shorter bundles in either or both developing or mutant (e.g. Eps8 KO mouse[36,49] cochlea tissues). Here we leverage our hair cell bundle segmentations to automate the measurement of bundle heights.

The steps for accurate and automated bundle height measurements involves calculating the distance from the tip to the base of the tallest row of stereocilia in hair cell bundles.

1. Iterate through each connected component in the labeled volume. Skip the background and any filtered components.
2. For each relevant component, identify all voxel coordinates that belong to the bundle.
3. Create a binary sub-volume for the current component where the component's voxels are marked as one, and all others are zeros.
4. Project the binary sub-volume along the z-axis to reduce it to a 2D projection by summing along the z-dimension and then applying a threshold to ensure the projection remains binary (values greater than 1 are set to 1).
5. Locate the highest (tip) and lowest (base) xy-coordinates in the 2D projection that have nonzero values:

- Find the highest point by identifying the minimum xy-coordinate value that corresponds to 1 in the projection.

- Find the bottom-most point by tracing downward from the centroid of the projection until reaching a xy-coordinate with a value of 0, then stepping back to the last non-zero coordinate.

6. Determine the z-coordinates of these points in the original 3D volume:

- For the highest point, find the z-coordinates where the voxel at the identified (x, y) location is 1.

- For the lowest point, determine the z-coordinate by identifying the first z-slice that contains any part of the component.

7. Store the coordinates of the highest and lowest points. These are used for calculating the 3D Euclidean distance between the tip and the base of the bundle.

8. Calculate the distance using the Euclidean distance formula between the stored highest and lowest points.

$$\text{Distance} = \sqrt{(x_2 - x_1)^2 + (y_2 - y_1)^2 + (z_2 - z_1)^2}$$

where (x1, y1, z1) and (x2, y2, z2) are the coordinates of the first and second points in these dimensions. Note that a scaling factor should be used for each dimension to ensure the calculated distance accurately reflects the true spatial separation between the two points in real physical units.

3.8 Utilizing pre-trained ResNet50 for targeted classification of cochlear tonotopic regions

For training and validation, our dataset comprises 36 3D stacks from the BASE region containing 535 images, 35 3D stacks from the MIDDLE with 710 images, and 38 3D stacks from the APEX with 651 images. For testing the model, we used ten stacks from BASE, nine from MIDDLE, and ten from APEX that were withheld from the training data. The classification of each stack as BASE, MIDDLE, or APEX is determined through a majority voting mechanism applied across all frames of the associated stack, starting from the median and extending over a length of 13 frames.

In this study, we employed a modified ResNet50 model[46] using the PyTorch framework[50] to classify images of cochlear regions into three categories: BASE, MIDDLE, and APEX, which correspond to the high, middle, and low frequency response tonotopic positions of the cochlear spiral. The model, initialized with weights from pre-trained networks, was adapted to our specific task by altering the final fully connected layer to output three classes. When using a pre-trained ResNet50 model, the weights of the model have been adjusted based on its training on ImageNet[51], contains over 14 million images categorized into over 20,000 classes, where it has likely learned rich feature representations for a wide variety of images. This pre-training makes the model a strong starting point for most visual recognition tasks. To enhance model robustness and adaptability, training involved dynamic augmentation techniques including random resizing, cropping, flipping, color adjustments, and rotations, followed by normalization tailored to the ResNet50 architecture. This approach utilized both frozen and trainable layers, allowing for effective feature extraction adapted from pre-trained domain knowledge while refining the model to the specific needs of our dataset. Training was conducted over 100 epochs with real-time monitoring via TensorBoard, optimizing for accuracy through stochastic gradient descent with momentum. The best performing model was systematically saved to achieve marked improvements in classification accuracy.

3.9 IHC and OHC row classification

We implemented a deep learning strategy employing multi-class classification to precisely identify and categorize each hair cell bundle as either IHCs (first row from the buttom), OHC1 (second row from the buttom), OHC2 (third row from the buttom), and OHC3 (forth row from the buttom).

For the training and validation process, we utilized the same dataset used for the 3D segmentation. Each dataset was manually annotated into four rows: IHC, OHC1, OHC2, and OHC3. We employed a multi-class segmentation approach using a U-Net architecture with a ResNet-50 backbone and a feature pyramid network. Transfer learning was utilized, leveraging pre-trained ImageNet weights to enhance model robustness. Given the existing 3D segmentation, our objective was simply to identify which cell corresponds to which row. To this end, we applied maximum projection to all frames in the stack to simplify and standardize the results.

Data augmentation has played a crucial role in enhancing the model's ability to generalize across different laboratory datasets, see Fig 18. Specifically, we have rotated the images by 10 and 20 degrees to both the right and left. Additionally, we have employed segmentation masks to mask out the raw images, effectively eliminating the background. This step creates additional training samples that focus on the foreground, helping the model to generalize better to other datasets where stains may not highlight the background, ensuring more accurate segmentation in diverse experimental conditions. Additionally, a CSV file that records distances is updated to include the new classifications, ensuring that each cell's identity (whether IHC, OHC1, OHC2, or OHC3) is documented.

3.10 Toward developing a foundational model for cochlea research: Integrating diverse data sources

For the final model available to the ear research community, we trained the architecture using a comprehensive dataset. This dataset included all the data from P5 (young mice), which consists of 45 stacks, as well as 10 stacks from P21 (adult mice), and 22 stacks provided by the Liberman lab. This amounted to a total of 901 2D images and 29,963 instances.

Given that this model is trained on young and adult mice of the same strain, as well as data from a different laboratory with varying settings, stains, and microscopy techniques, we consider this model to be the first trial in developing a foundational model. It is hoped that this model will work with data from other laboratories without requiring fine-tuning.

We are committed to maintaining our GitHub repository and actively encourage collaborators from other labs to share their data. By doing so, we aim to broaden the model's applicability and enhance its robustness, ultimately benefiting the entire ear research community.

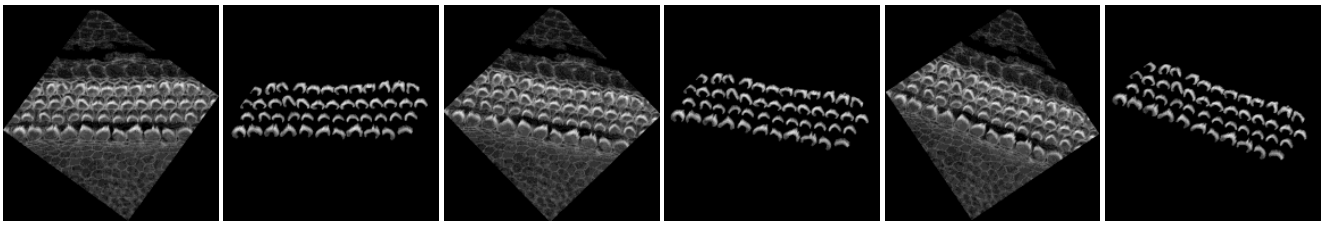


Figure 18. Augmentation of maximum projection images from 3D confocal stacks for training a classification model that discriminates between IHC, OHC1, OHC2, and OHC3.

3.11 Computational resources for all the experiments

All experiments were conducted on a local desktop computer equipped with a 13th Gen Intel(R) Core(TM) i9-13900K CPU, 128GB of RAM, and an NVIDIA GeForce RTX 4080 GPU with 16.0 GB of dedicated memory, running Microsoft Windows 11 Pro. We utilized the PyTorch framework[50] to implement all machine learning models to develop the Napari plugin. For training using the Facebook Research Detectron2 library[44], we utilized the Windows Subsystem for Linux (WSL), as the library is not supported natively on Windows. To integrate the algorithm into the Napari plugin[12], we built an executable that runs through one of the plugin's buttons via WSL. Consequently, users will need to set up WSL to utilize this plugin, details of which are thoroughly described in our GitHub documentation.

3.12 Data and Code Availability

In support of open science and to enhance reproducibility, we are pleased to publicly release all annotated datasets used for training and testing in our experiments. This dataset consists of 1,870 stereocilia bundles (410 IHCs and 1,460 OHCs) from P5 mice, and 335 bundles (92 IHCs and 243 OHCs) from P21 mice. It is freely available to the research community, facilitating further studies, benchmarking, and advances in the field.

Additionally, the complete source code for Napari plugin, has been made publicly accessible. The code can be accessed through our dedicated GitHub repository at the following URL:

https://github.com/ucsdmanorlab/Napari-VASCilia

The full documentation is accessible through this link:

https://ucsdmanorlab.github.io/Napari-VASCilia/

We encourage the community to utilize these resources in their research and to contribute improvements or variations to the methodologies presented.

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Acknowledgements

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Author contributions statement

Yasmin Kassim was responsible for the plugin design, fully implemented all functions in Python, wrote the manuscript, proofread the ground truth data, created all figures, and established the GitHub repository and codebase. David Rosenberg acquired most of the images for training data. Samprita Das and Alma Renero were responsible for the height bundle ground truth analyses. Samia Rahman, Ibraheem Al Shammaa, Samer Salim, Zhuoling Huang, and Kevin Huang, undergraduate students, manually annotated the ground truth stereocilia bundle segmentations using the CVAT annotation tool. Yuzuru Ninoyu assisted with some of the imaging data, with Rick Friedman's supervision and support. Artur Indzhykulian provided additional imaging data for testing. Cayla Miller contributed to the review of the manuscript. Uri Manor, the Principal Investigator, conceived and supervised the project, and provided critical revisions and updates to the manuscript.

Supplementary Materials

VASCilia workflow software plugin screenshots, illustrating all steps and buttons

1. Open Cochlea Datasets (CZI, LIF, TIF) and Preprocess button:

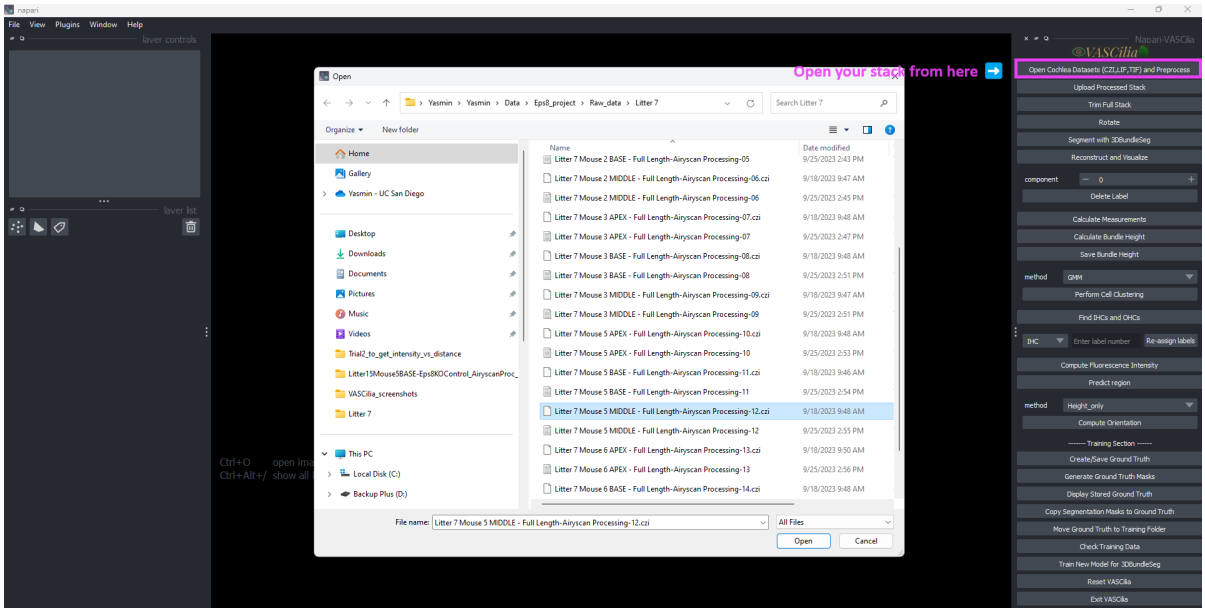

Figure 19. Screenshot of the software's opening screen, showcasing the initial interface and available options. The software supports three formats: CZI, LIF, and TIF.

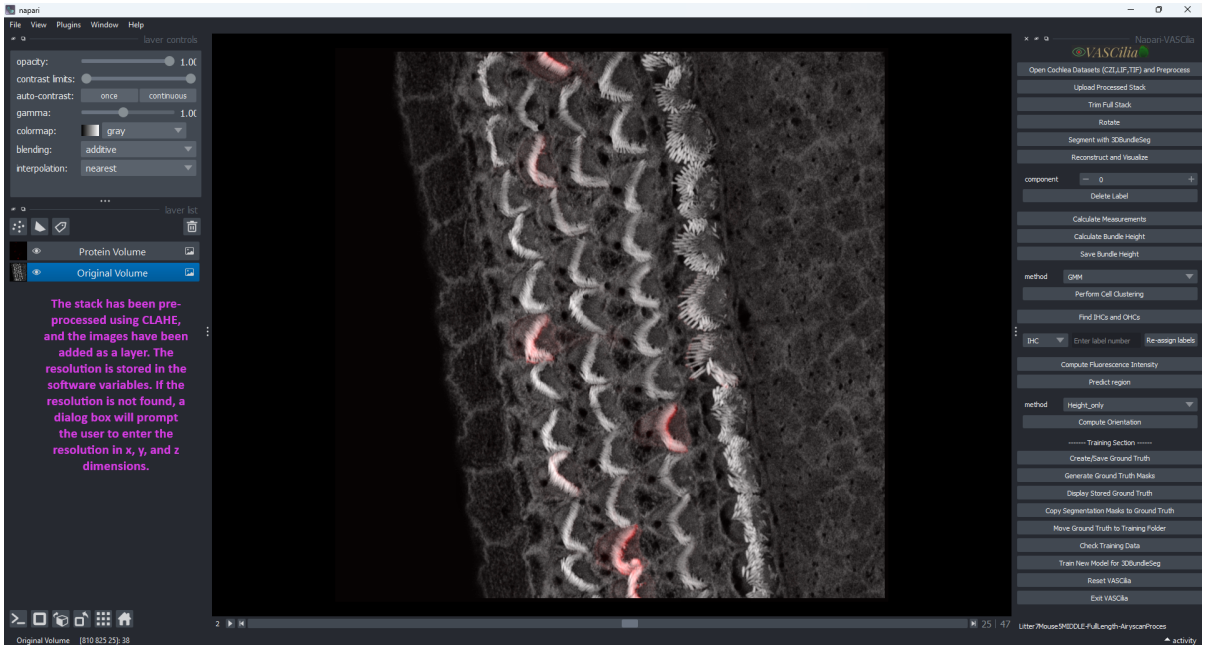

Figure 20. Screenshot of the software's opening screen, displaying the stack as a layer.

2. Upload Processessed stack button:

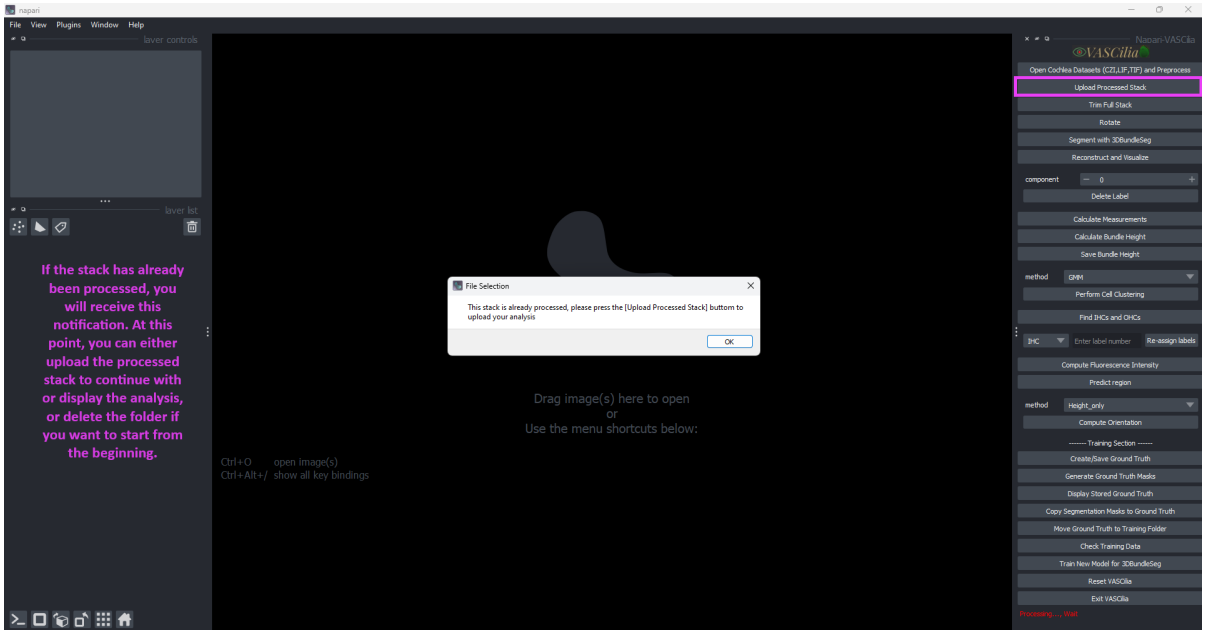

Figure 21. Screenshot of the software's opening screen showing that the stack is already opened and processed.

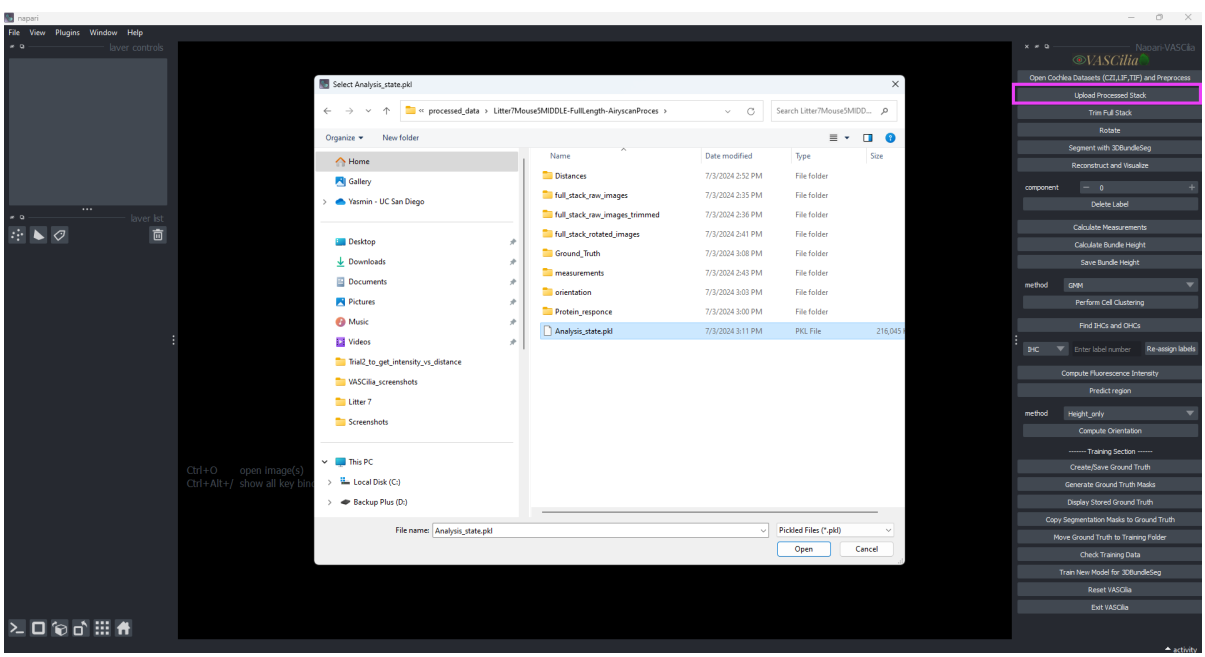

Figure 22. Screenshot of the software's screen showing that the processed stack can be uploaded from the pickle file.

3. Trim Full Stack button:

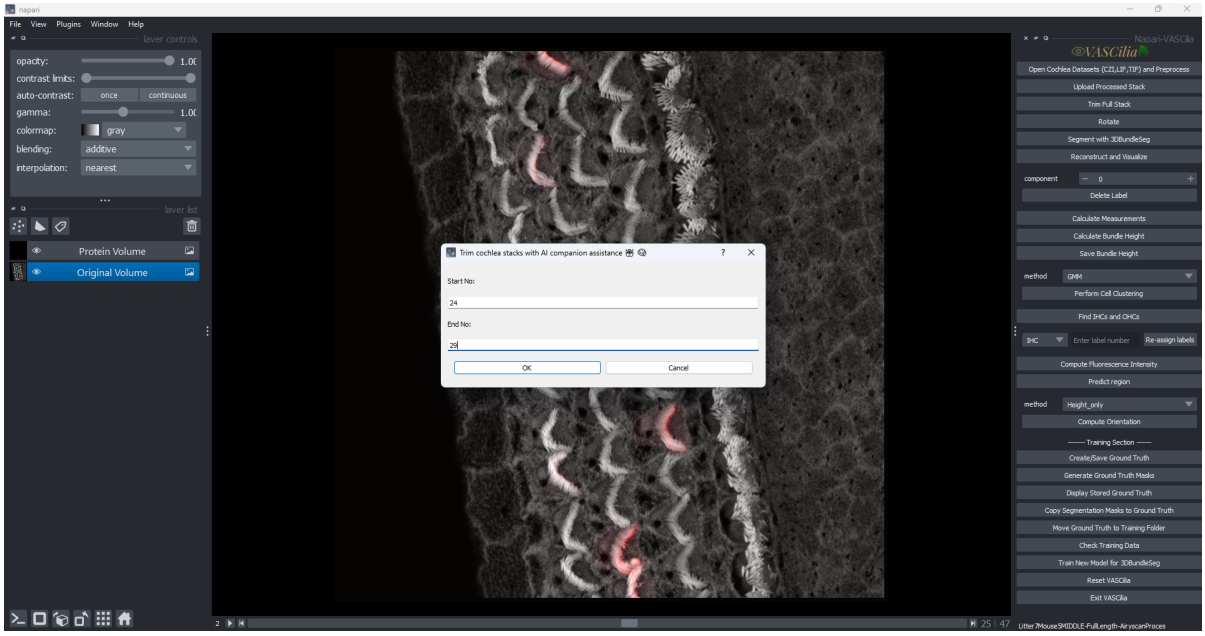

Figure 23. A screenshot of the software's screen demonstrating how the automated model ZFT-Net provides preliminary suggestions for trimming, while still allowing the user to manually select the slices of interest.

4. Rotate button:

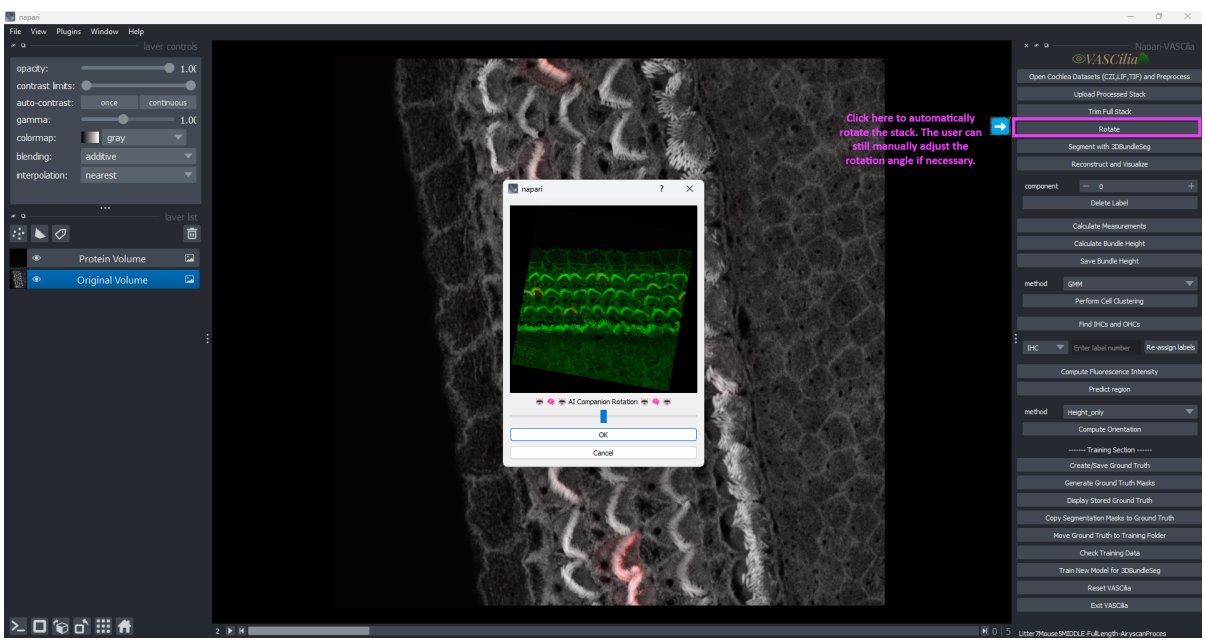

Figure 24. Screenshot of the software's screen for rotating the stack using the trained model, while still allowing the user to manually correct the orientation angle if necessary

5. Segment with 3DBundleSeg button:

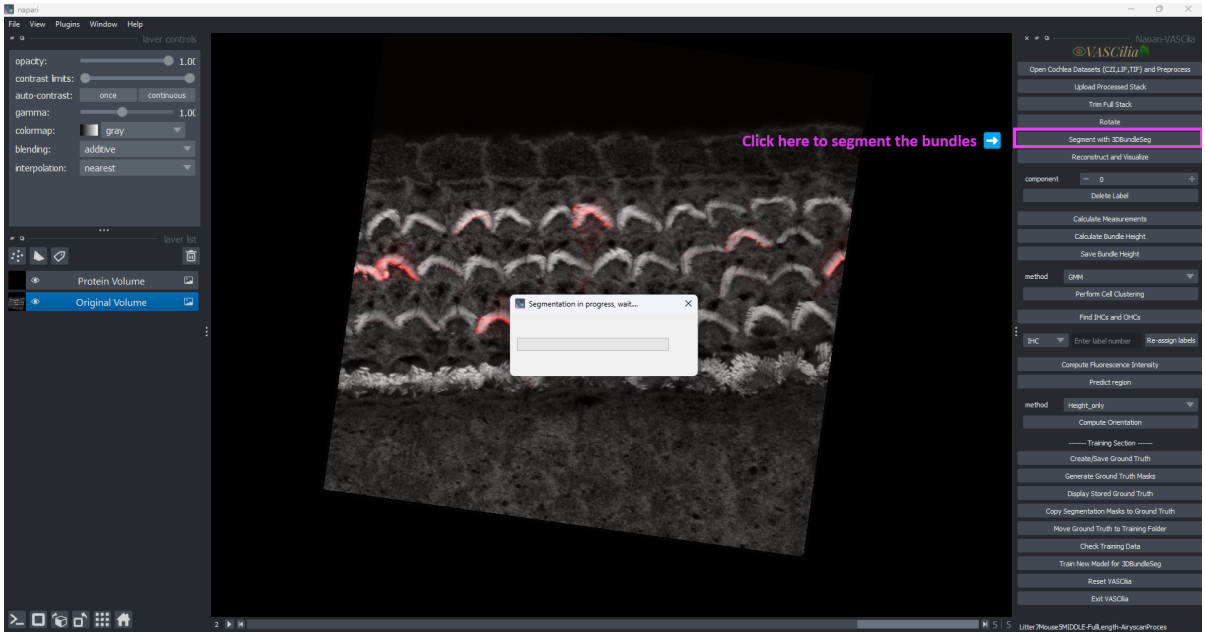

Figure 25. Screenshot of the software's screen for segmenting the stack.

6. Reconstruct and Visualize button:

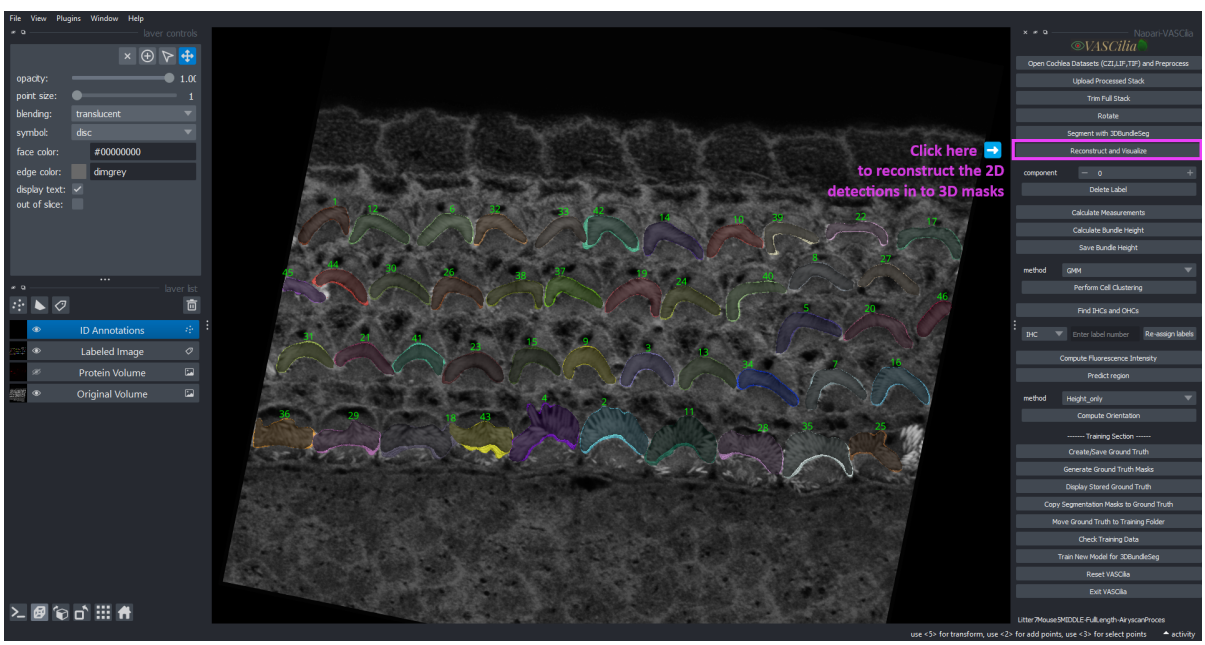

Figure 26. Screenshot of the software showing the bundle segmentation results.

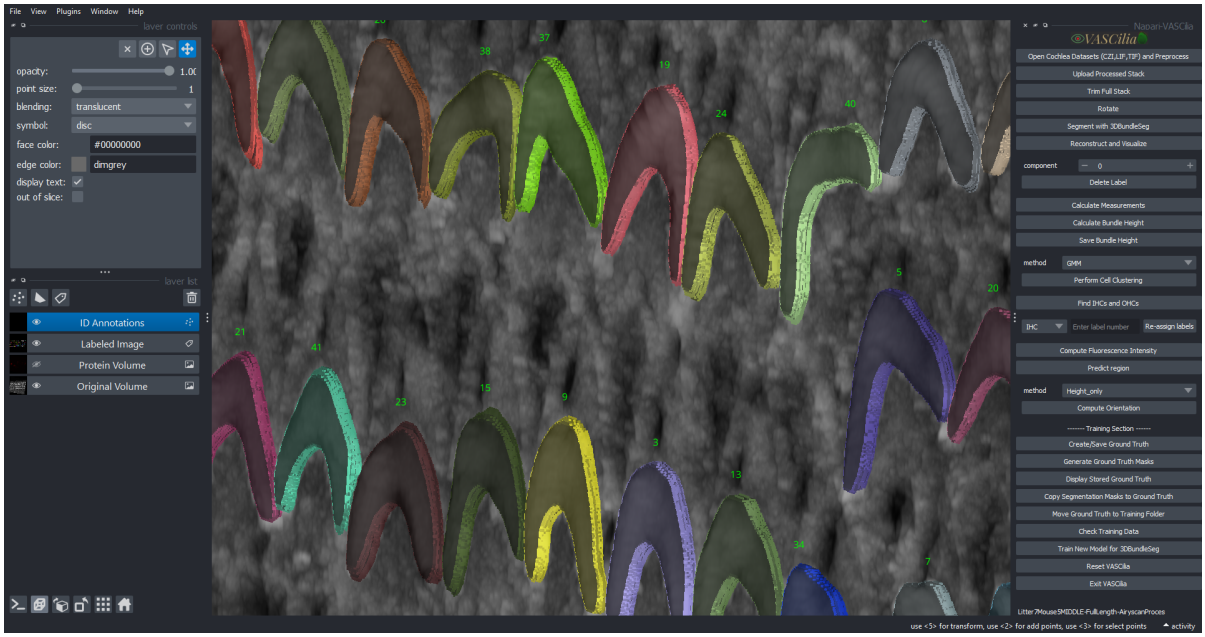

Figure 27. Screenshot of the software with a zoomed-in view of bundle segmentation, showing each segmented bundle labeled with an ID.

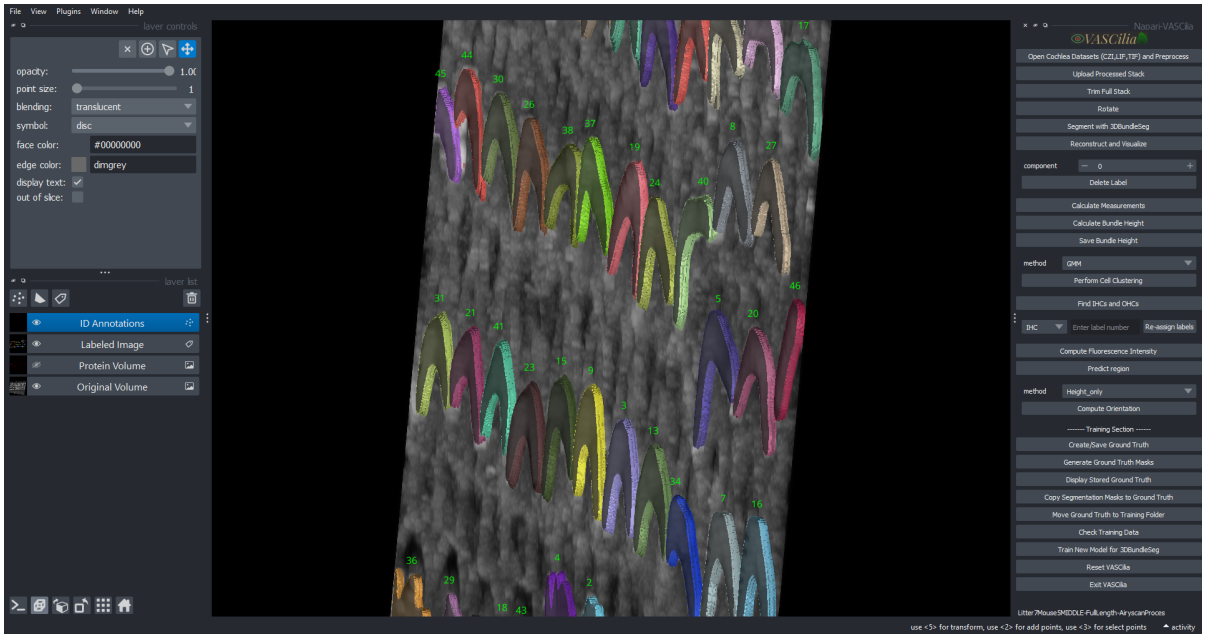

Figure 28. Screenshot of the software with bundle segmentation while the user is rotating the layer.

7. Delete button:

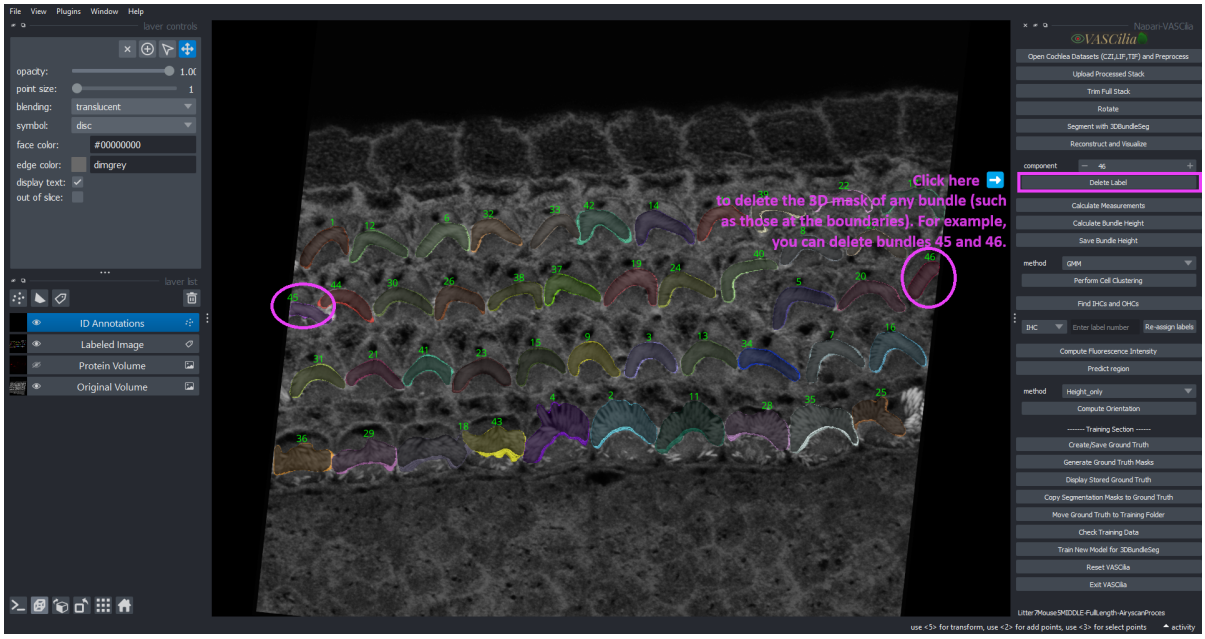

Figure 29. Screenshot of the software with the delete action.

8. Calculate measurements:

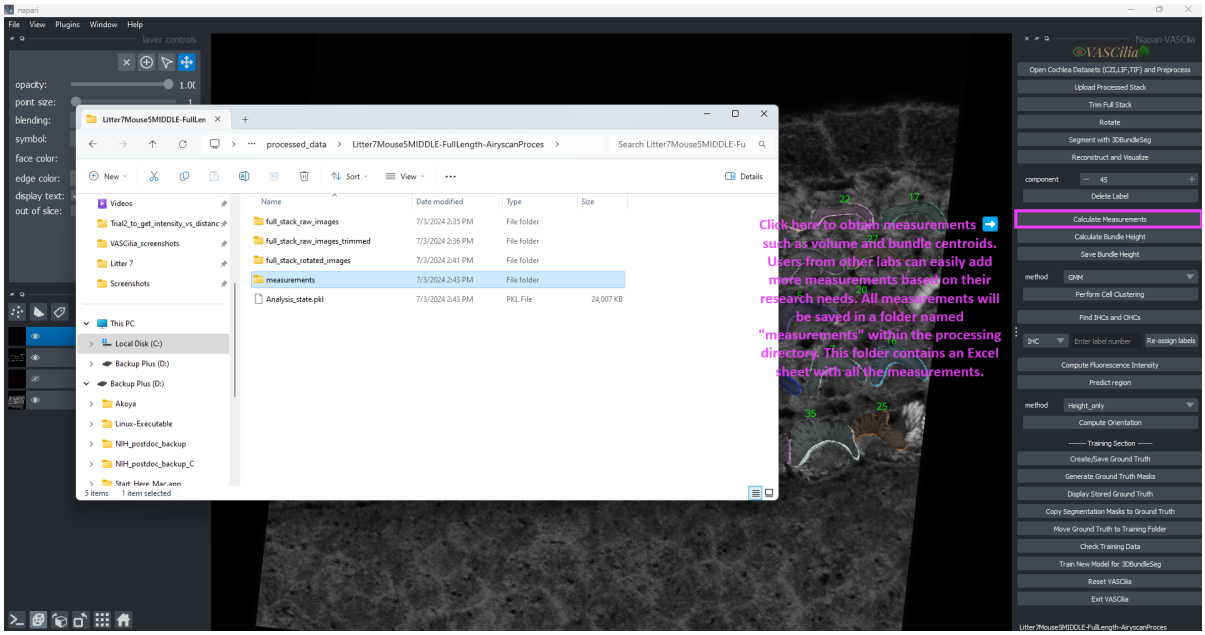

Figure 30. Screenshot of the software with measurements calculation action.

9. Calculate Bundle Height and Save Bundle Height buttons:

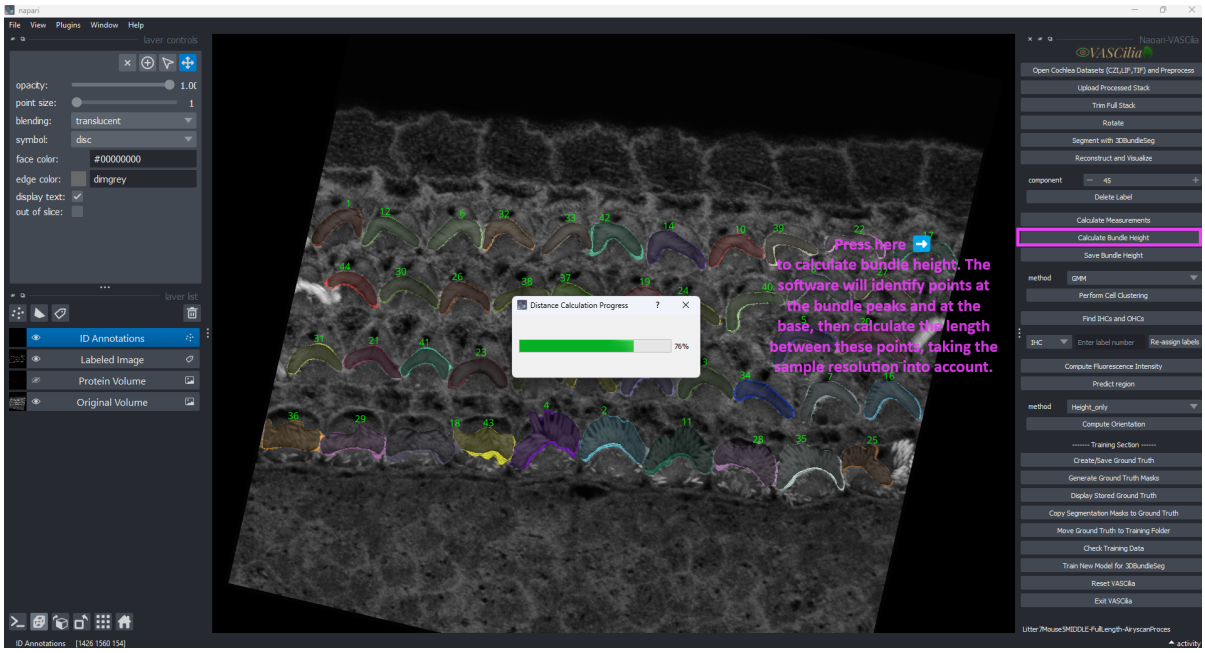

Figure 31. Screenshot of the software with the bundle height calculation action.

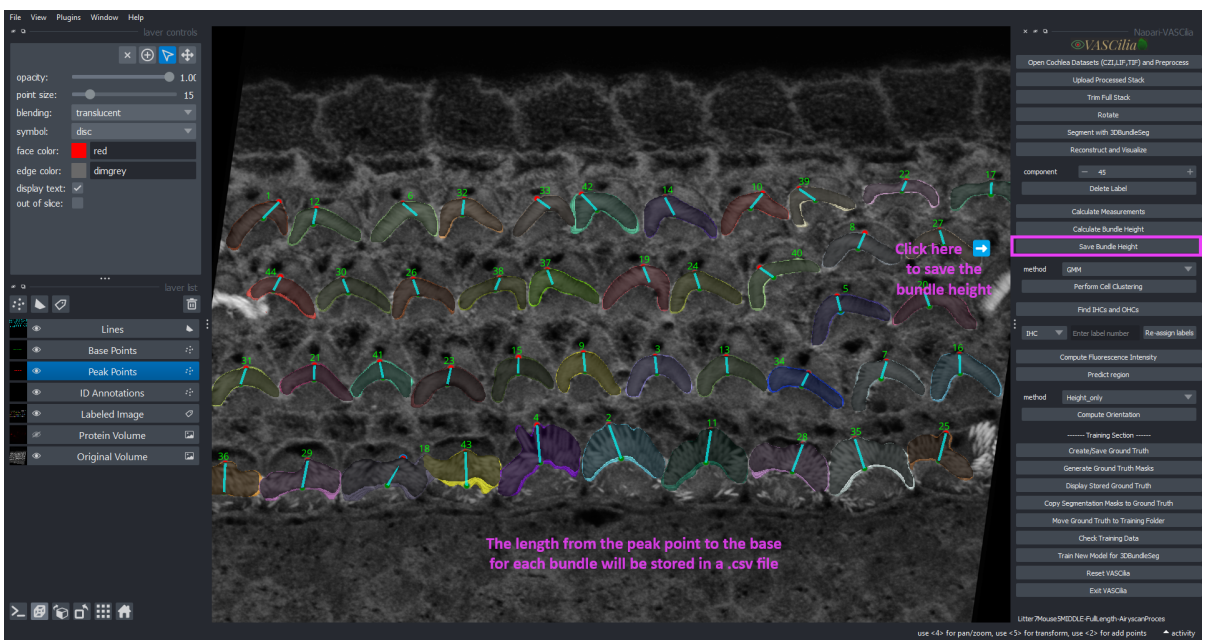

Figure 32. Screenshot of the software with the bundle height action, showing the peak points in red, base points in green, and the line connecting them in cyan.

10. Perform Cell Clustering button:

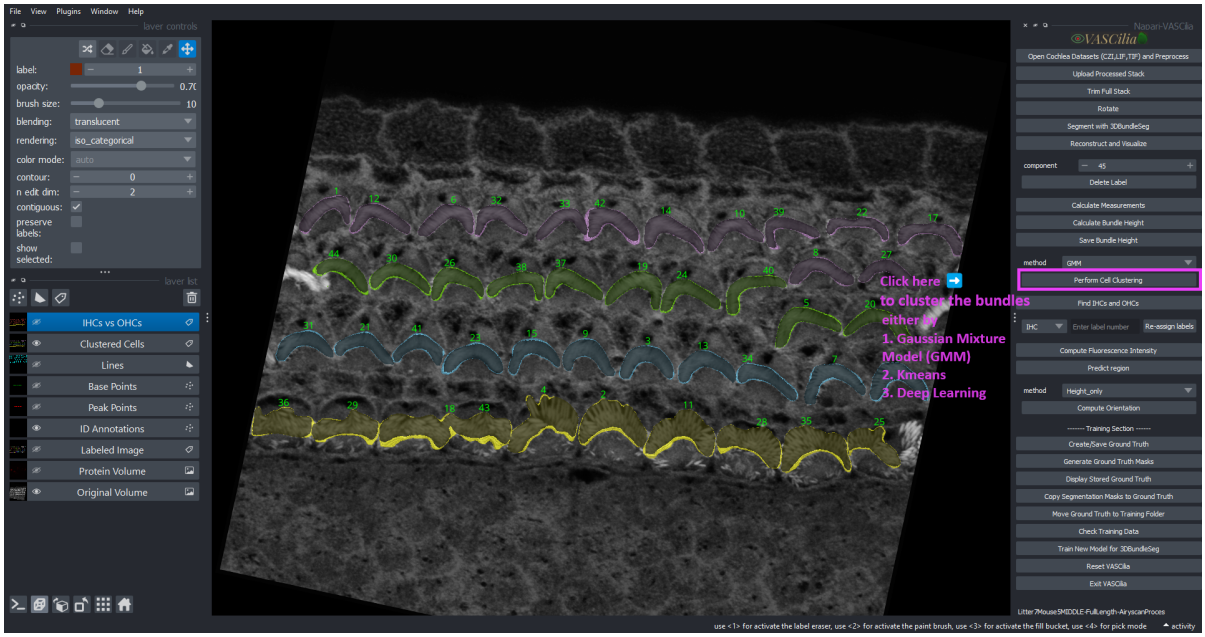

Figure 33. Screenshot of the software with the cell type identification button and Gaussian Mixture Model (GMM) results.

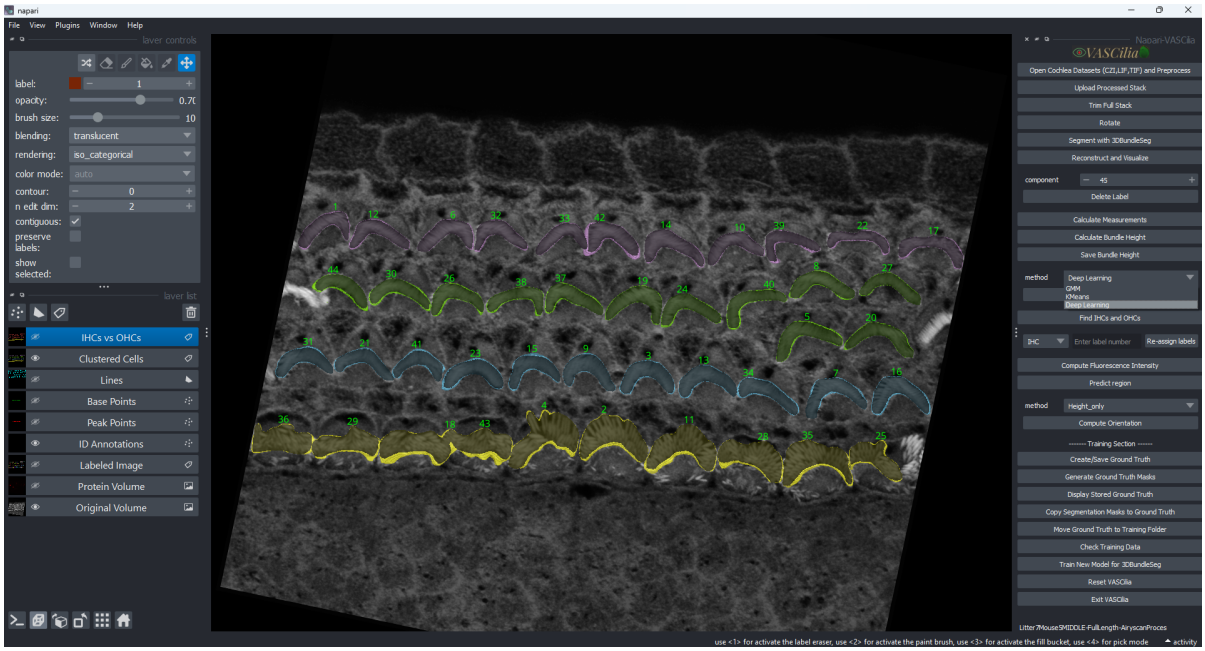

Figure 34. Screenshot of the software with the cell type identification button and Deep Learning results.

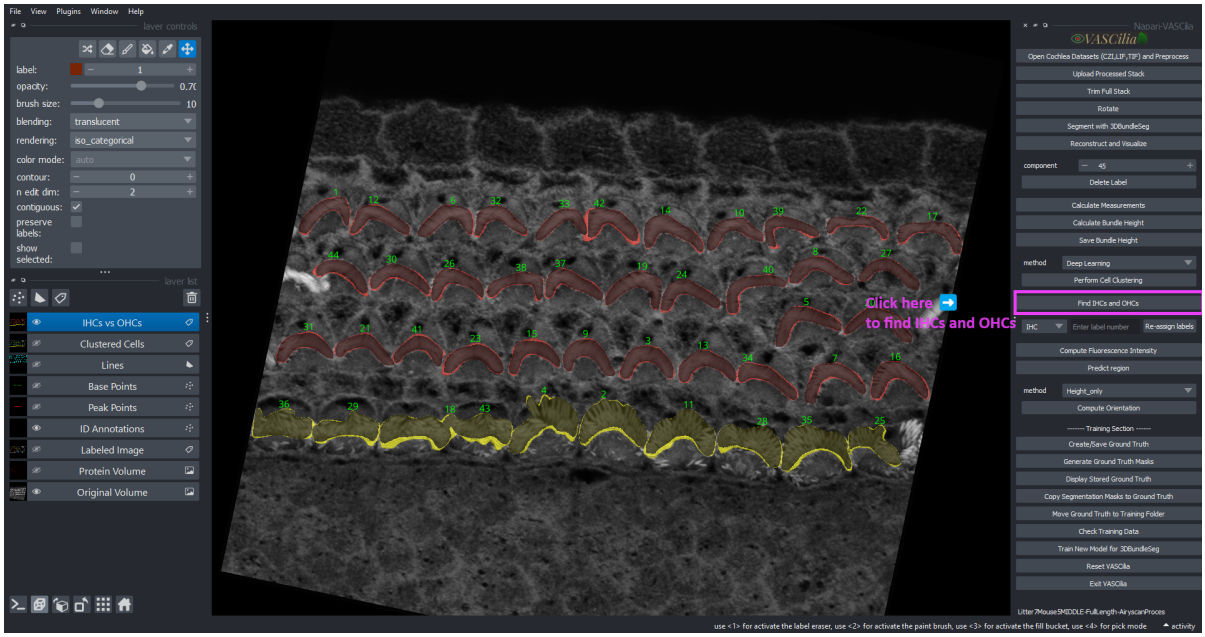

Figure 35. Screenshot of the software with the cell type identification process to distinguish between IHCs and OHCs.

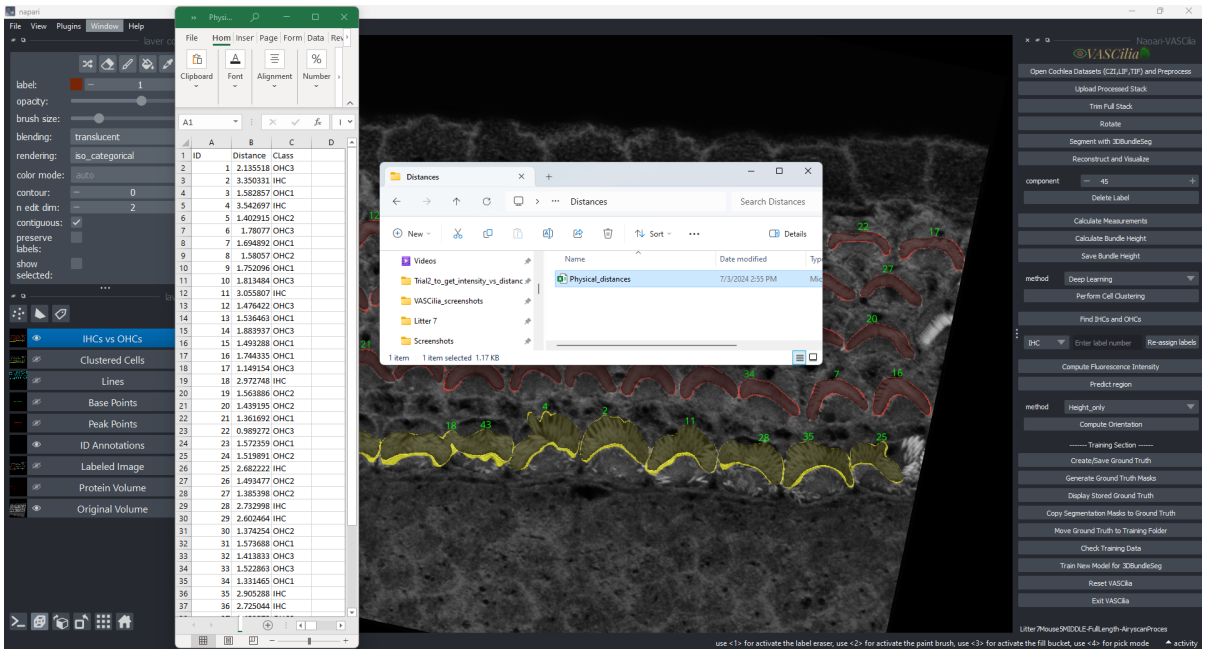

Figure 36. Screenshot of the software showing the distance.csv file updated with a new column called Class based on the cell type identification results.

11. Compute Fluorescence Intensity button:

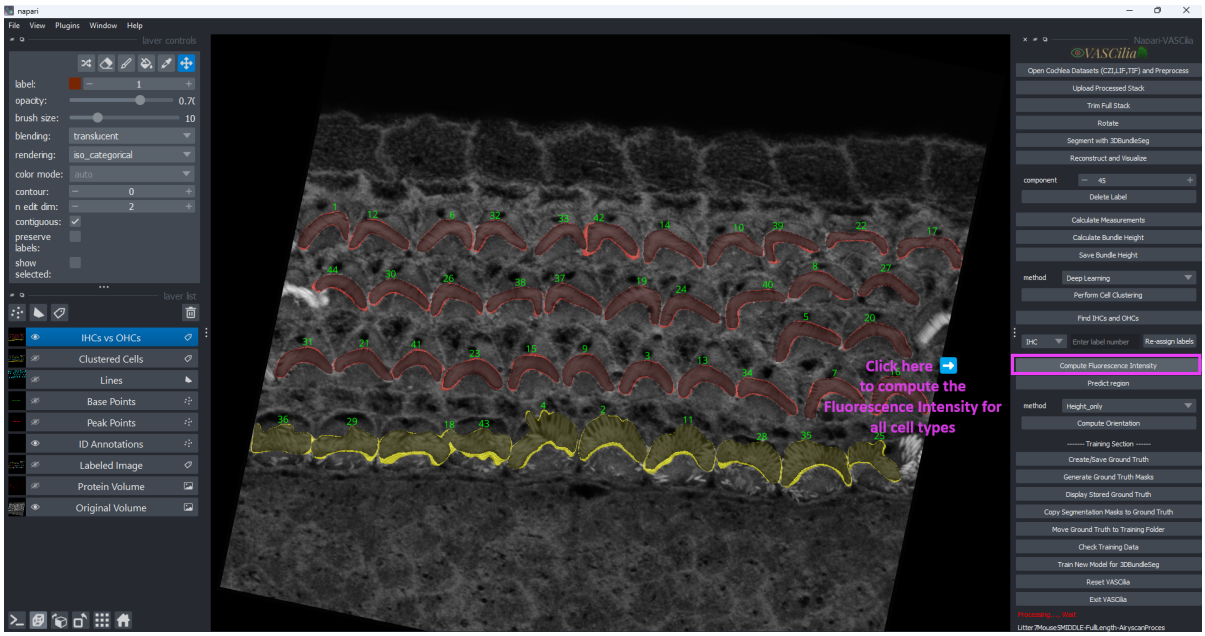

Figure 37. Screenshot of the software with the Compute Fluorescence Intensity action.

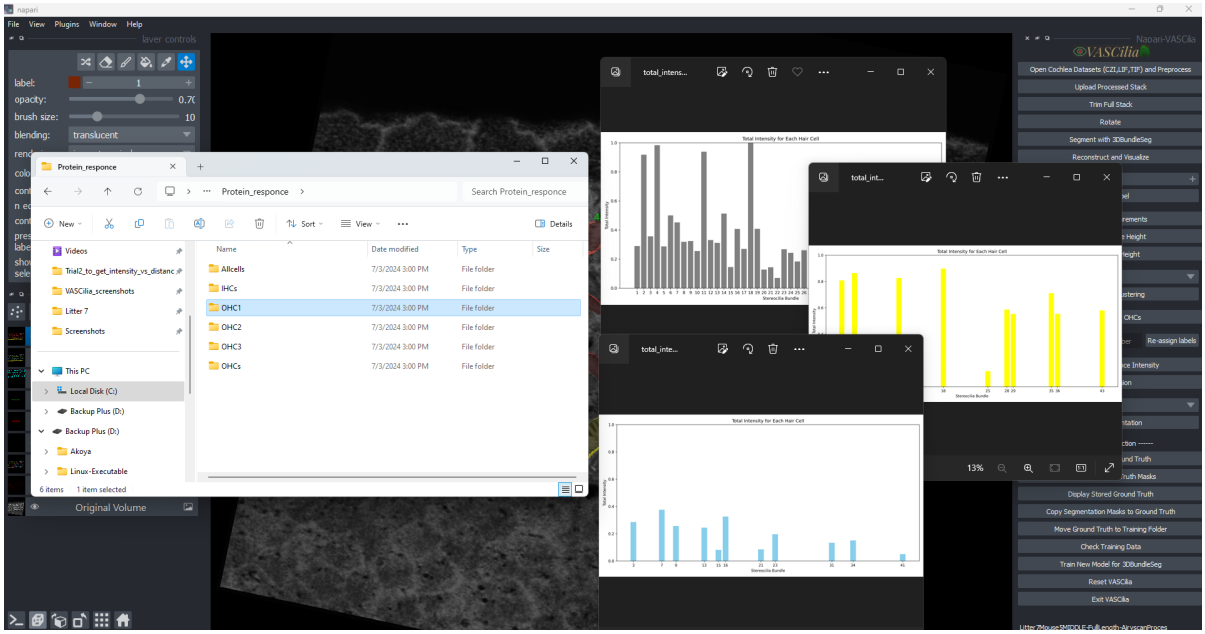

Figure 38. Screenshot of the software with the Compute Fluorescence Intensity action showing the bar plots for each cell type and the .csv file.

12. Predict Region button:

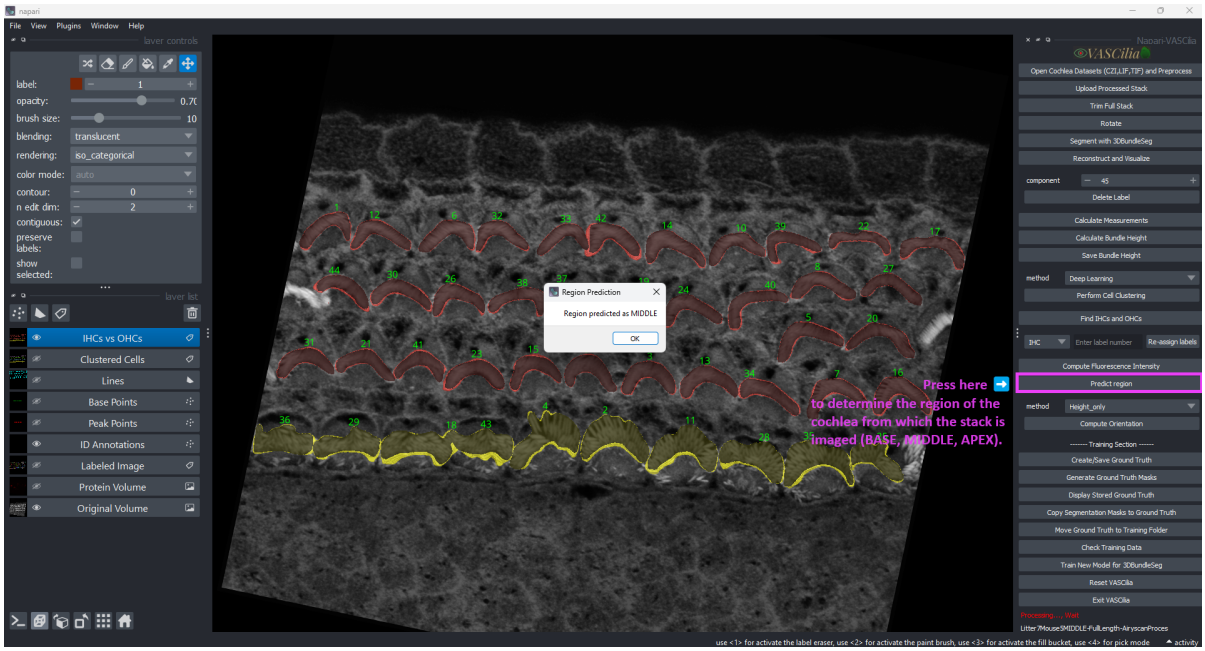

Figure 39. Screenshot of the software with the predict region action.

13. Compute Orientation button:

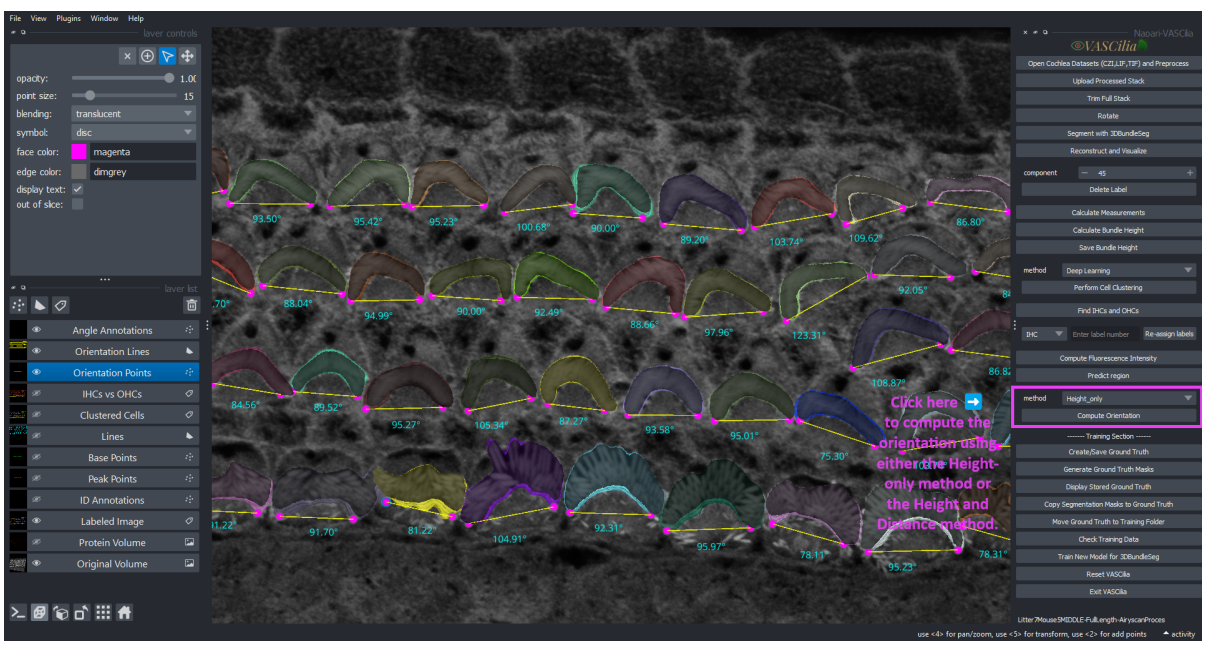

Figure 40. Screenshot of the software with the orientation measurement action.

14. Create/Save Ground Truth button:

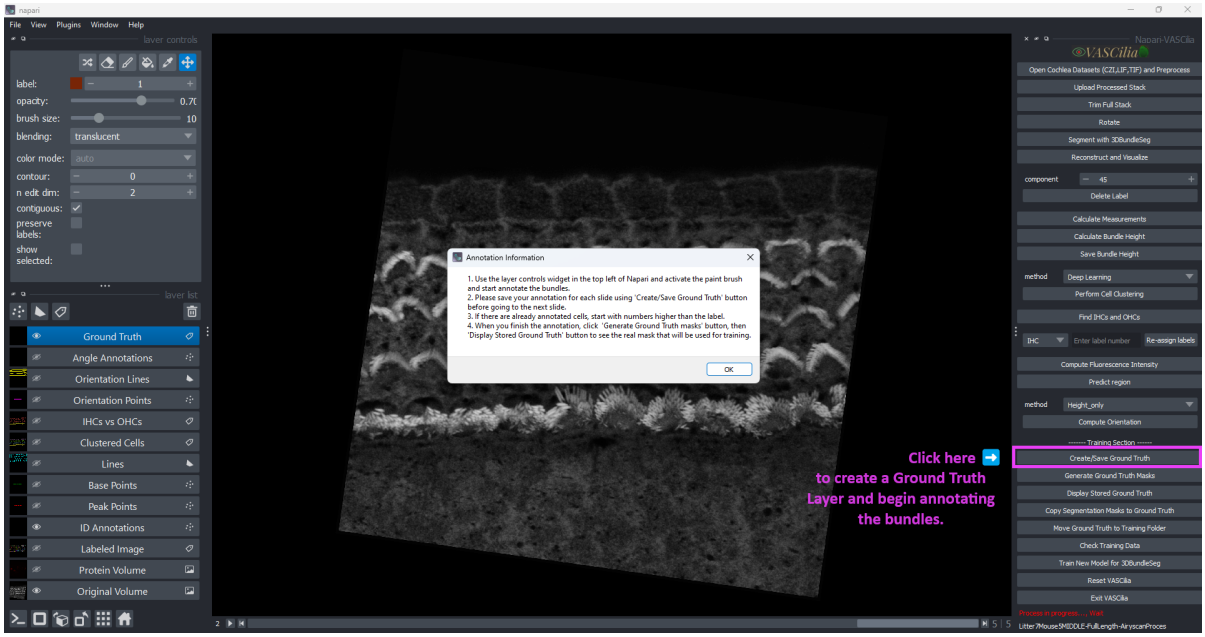

Figure 41. Screenshot of the software with the "Create GT" action

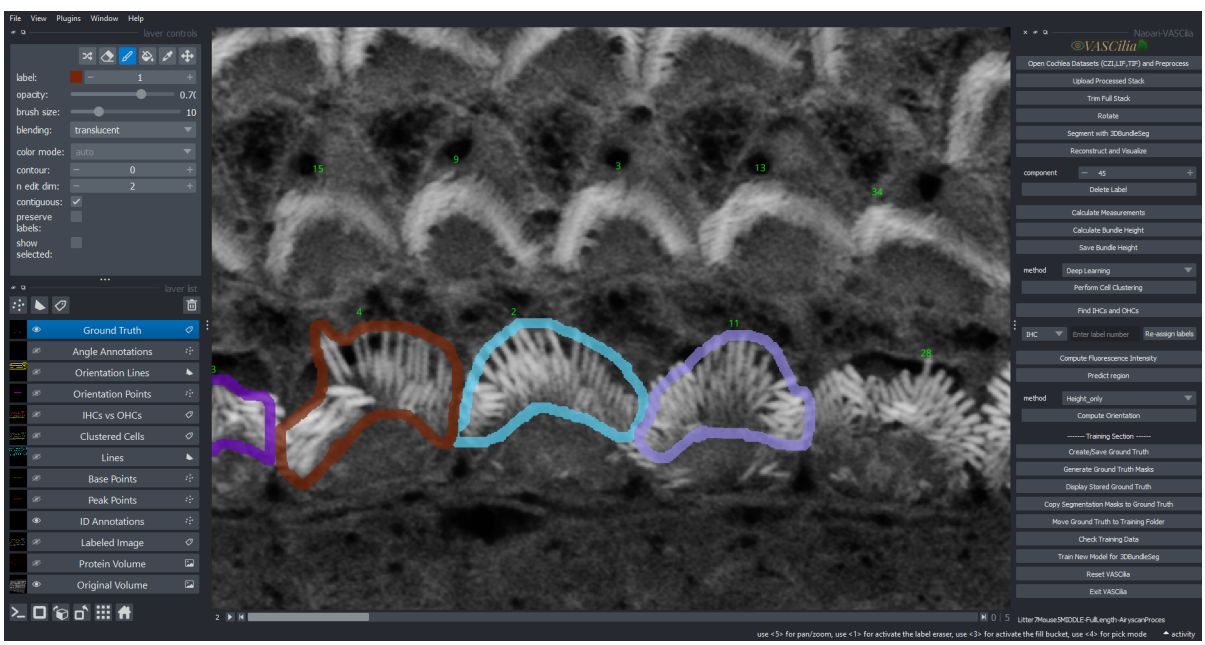

Figure 42. Screenshot of the software showing cells being annotated.

15. Display Stored Ground Truth button:

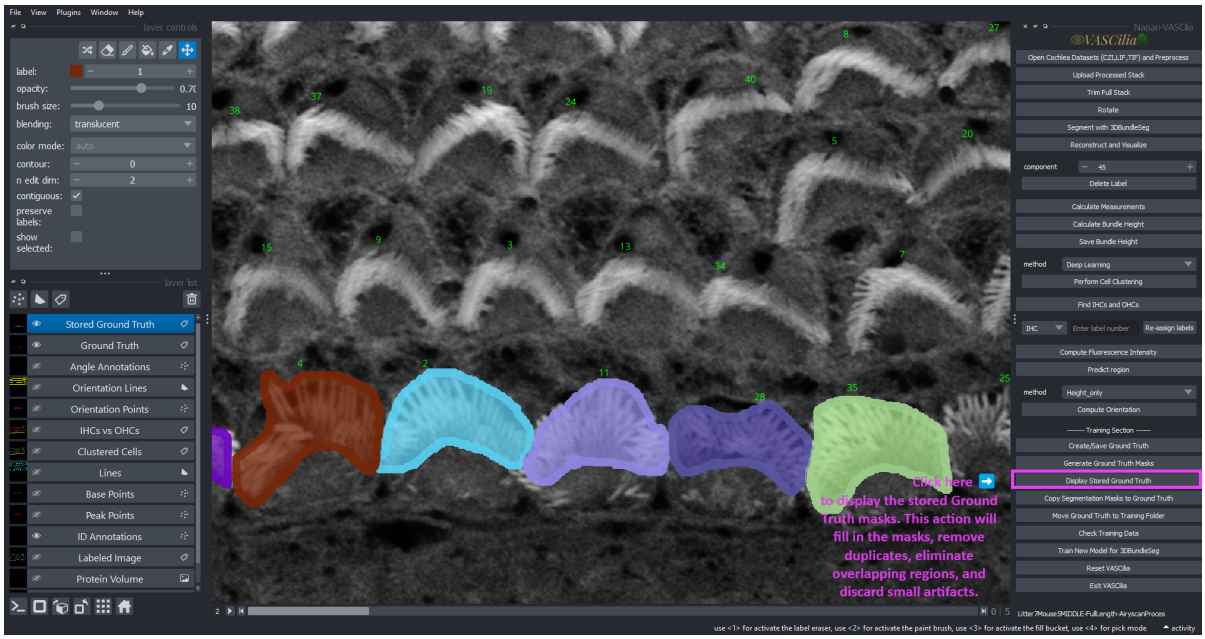

Figure 43. Screenshot of the software with the Display Stored Ground Truth action.

16. Copy Segmentation masks to Ground Truth button:

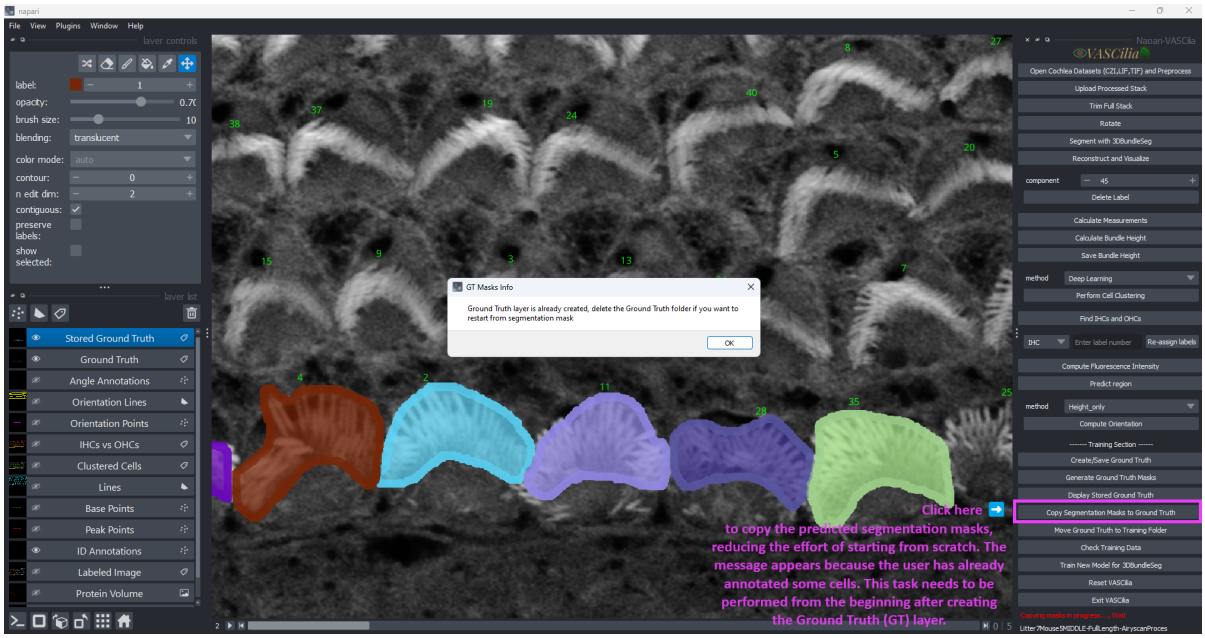

Figure 44. Screenshot of the software with the Copy Masks action, which copies the predicted masks to the GT layer.

17. Check Training Data and Train a new model for 3DBundleSeg:

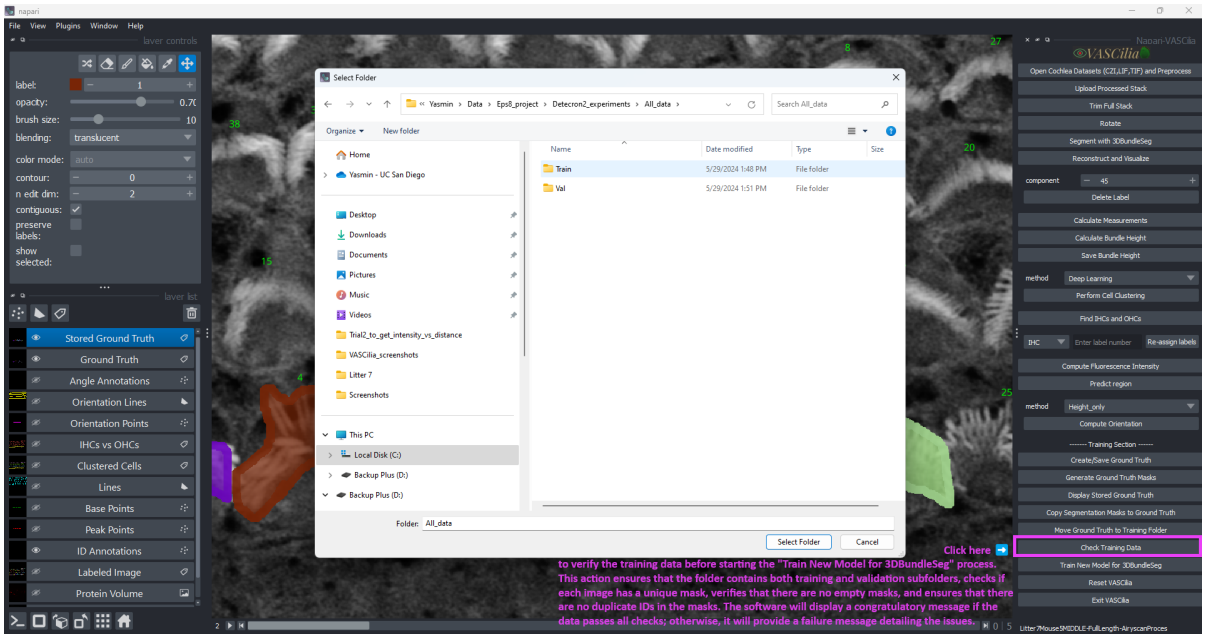

Figure 45. Screenshot of the software with the Check Training Data action.

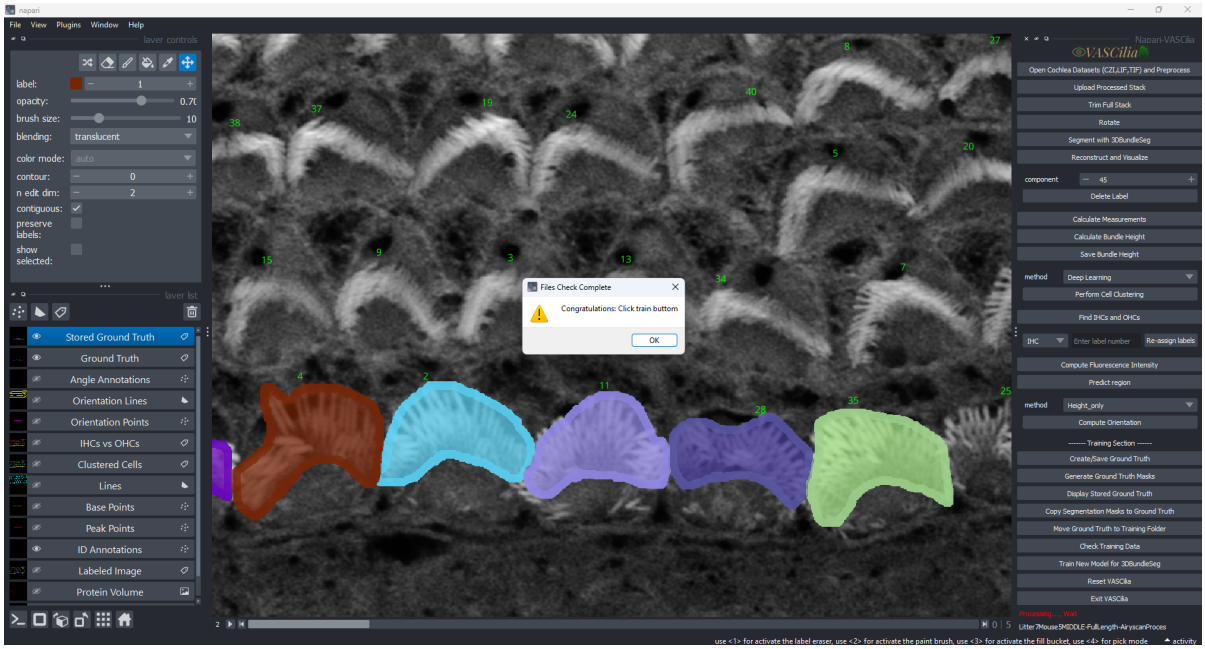

Figure 46. Screenshot of the software with the Check Training Data action button showing a Congratulation message.

18. Reset and Exit VASCilia:

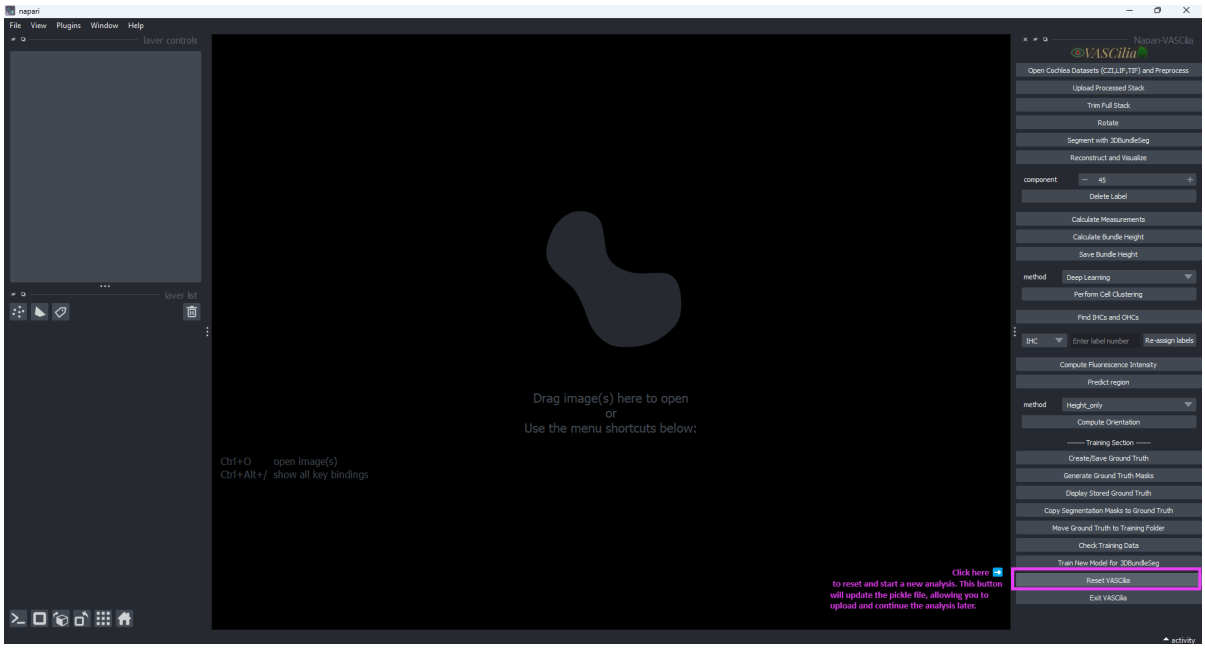

Figure 47. Screenshot of the software with the Reset button.

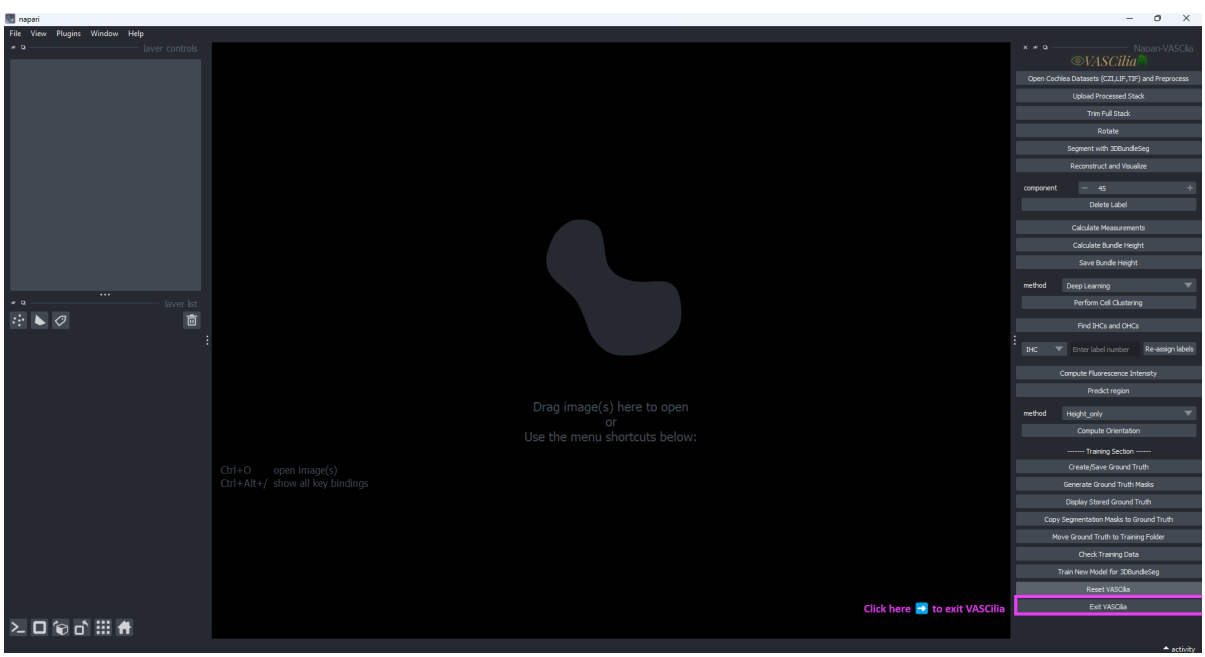

Figure 48. Screenshot of the software with the exit button.

VASCilia analysis for Artur Indzhykulian lab datasets: screenshots for various samples.

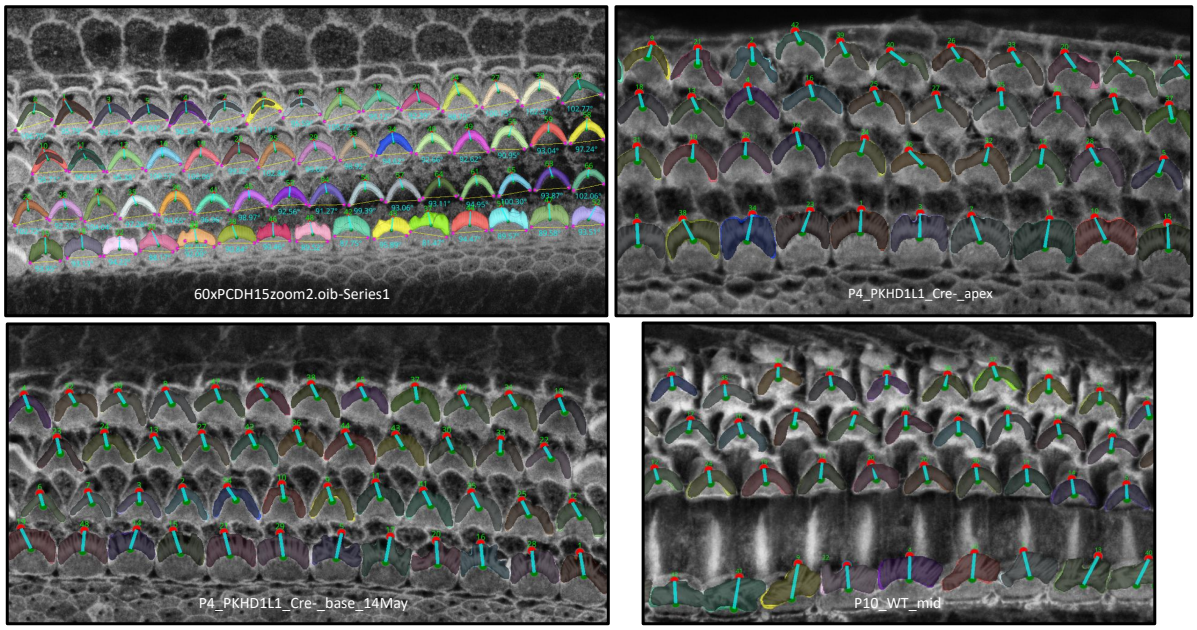

Figure 49. Screenshot of the software with different samples.

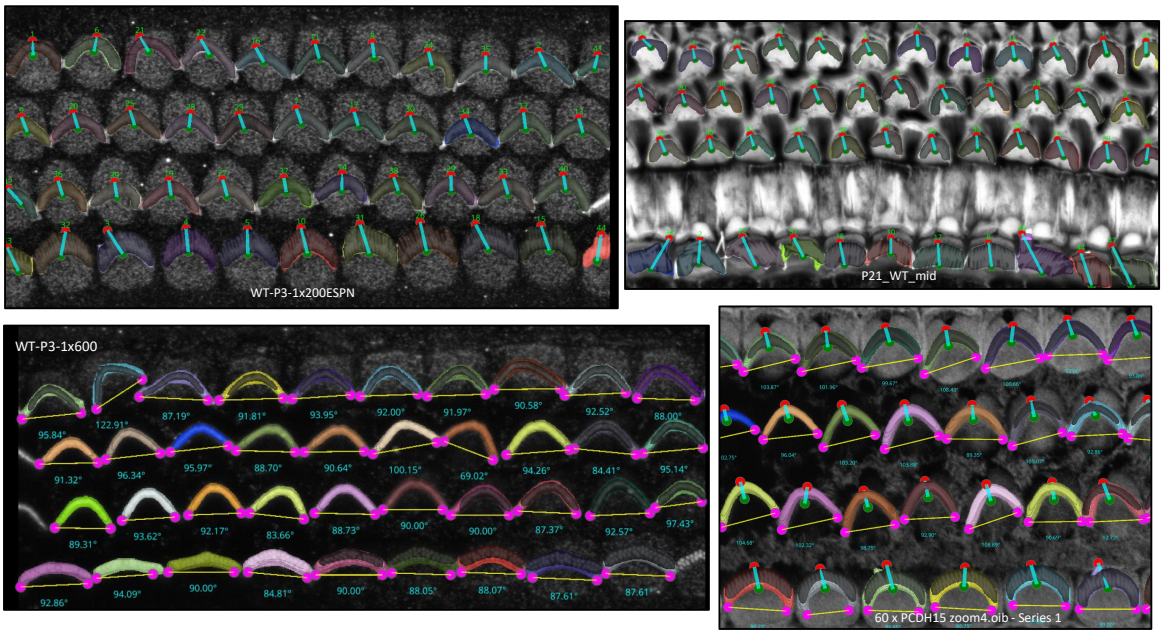

Figure 50. Screenshot of the software with different samples.

VASCilia analysis for Liberman lab datasets: screenshots for three samples.

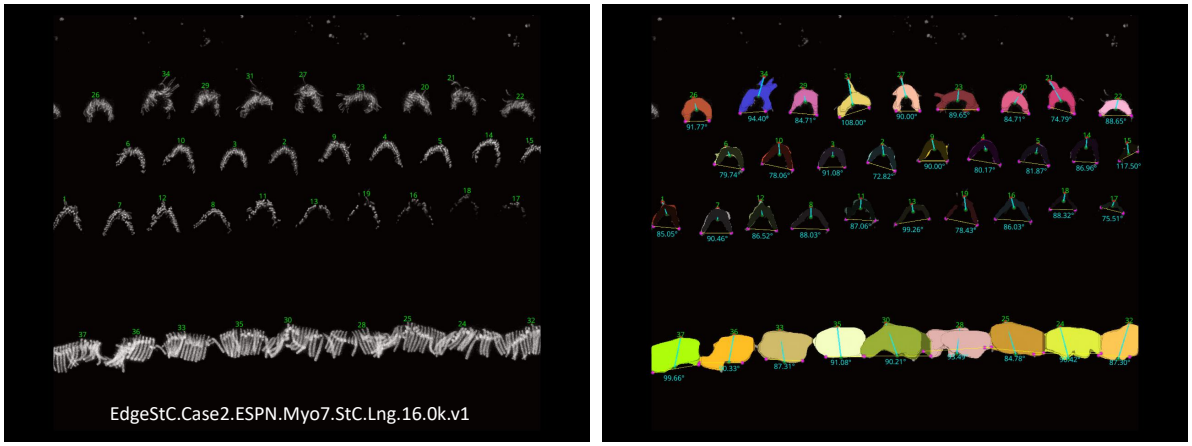

Figure 51. Screenshot of the software with Liberman lab mouse cochlea data.

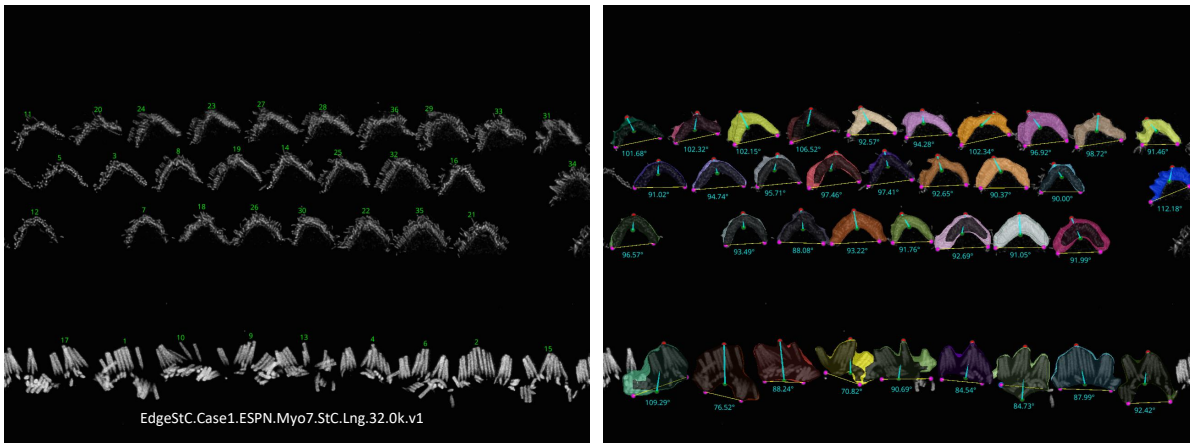

Figure 52. Screenshot of the software with Liberman lab mouse cochlea data.

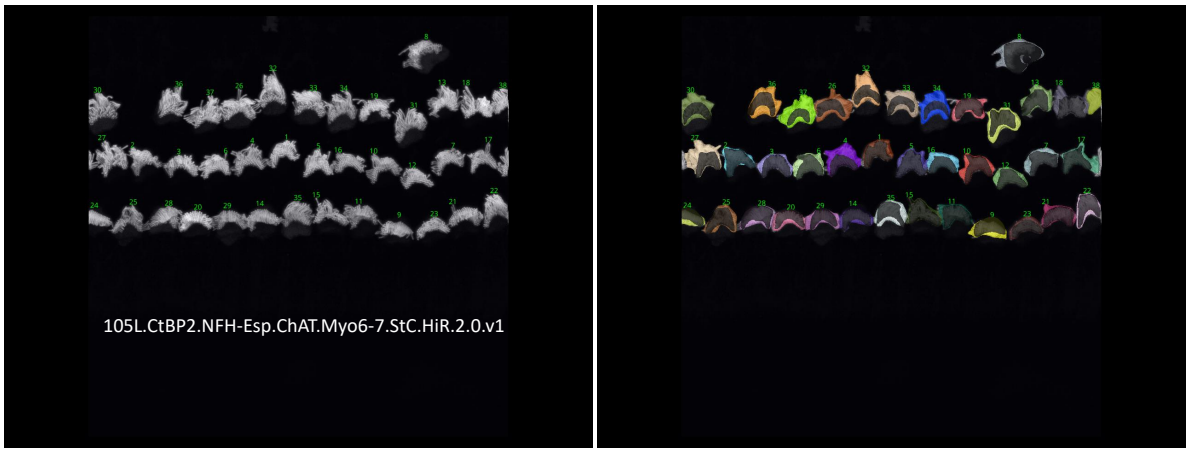

Figure 53. Screenshot of the software with Liberman lab human cochlea data.

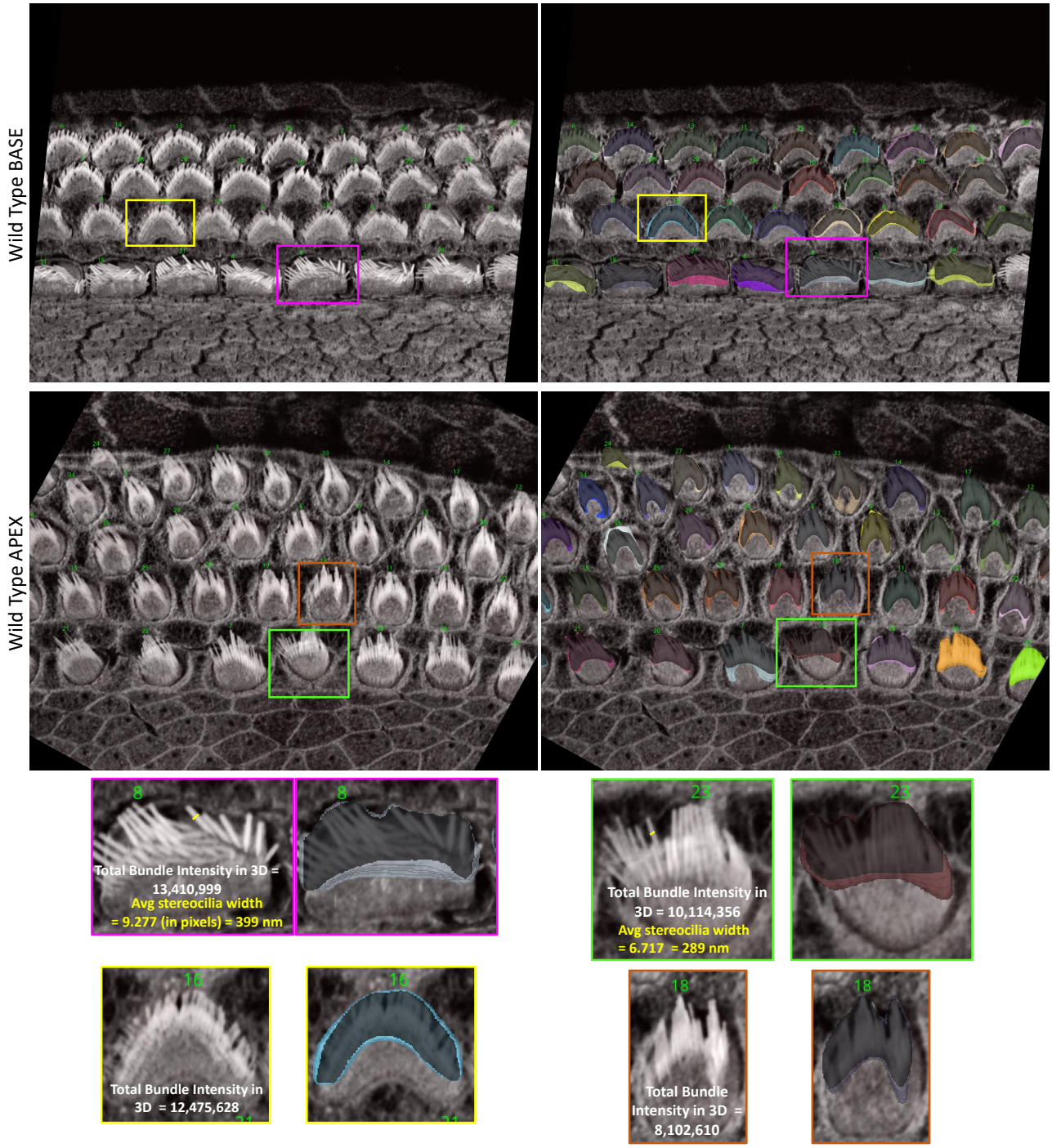

Figure 54. Visual comparison of base and apex bundles in WT animals reveals that the base appears stiffer, with wider stereocilia and bundles, while the apex displays finer stereocilia and tighter bundles, leading to a reduced phalloidin intensity signal.