From Image to Results

Organoid Analysis

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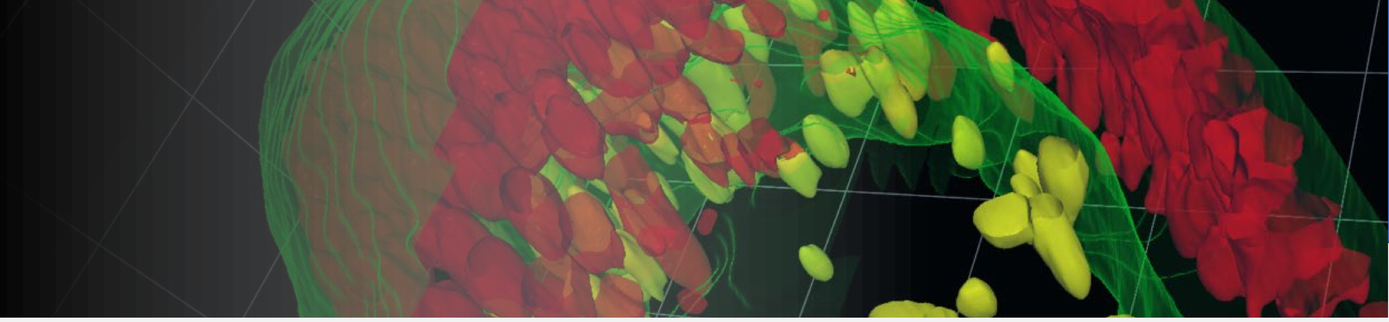


Introduction

Intestinal organoids have become indispensable tools for studying both normal gut development and mechanisms leading to morbidities e.g. Inflammatory Bowel disease. Intestinal organoids grow out from single intestinal stem cells. With the proper signaling cues applied, they eventually form organoids consisting of a single layer of enterocytes surrounding a hollow lumen that resembles the lumen of a real gut (Fig. 1A).

The Wnt pathway is a well-known signaling pathway regulating intestine development and maintenance. Wnt contributes to maintaining healthy tissue stem cells and the transition and differentiation of stem cells into mature enterocytes (intestinal tissue cells). On the other hand, excessive Wnt activity (e.g., by genetic mutations) contributes to intestinal cancer.

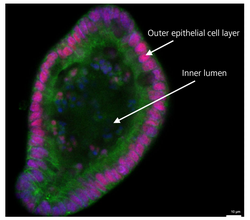


Fig 1A: **Basic morphology of an intestinal organoid.** A central cross-section of a complete organoid image set.

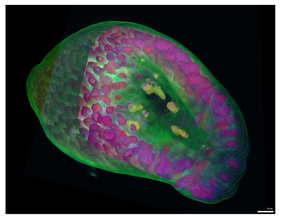


Fig 1B: **Segmentation of organoid.** Complete 3D organoid image set after analysis in arivis Vision4D software.

Objective

Microscopic imaging of organoids is a challenge due to their large size, light scattering characteristics, environmental requirements, generating large 3D data sets and phototoxicity. Therefore, the appropriate selection of the image acquisition tool is vital to acquire the highest quality data sets.

In this application story we showcase a simple imaging experiment performed on intestinal organoids treated with and without a Wnt-inhibiting drug, with the experimental goal to study the role of Wnt signaling in organoid formation.

Materials and Methods

- Intestinal stem cells were labelled with Histone2B-RFP and Mem9-GFP to mark cell nuclei and membranes.
- Isolated single intestinal stem cells were allowed to grow to organoids for 5 days in the presence or absence of Wnt Signaling Pathway inhibitor IWP-2.
- Organoids were then fixed and antibody-stained for Aldolase B, which is a marker for differentiated enterocytes, and counterstained with DAPI .
- Image acquisition was performed employing a laser scanning confocal ZEISS **Celldiscoverer 7 with LSM900 Airyscan 2 processing**.
- **arivis Vision4D** was used to carry out image processing and quantification of organoid samples.

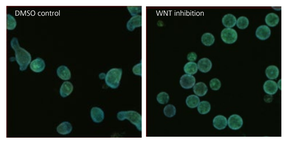


Fig 2A: **Overview image of organoids following control treatment (left) or Wnt inhibition (right).** Note differences in morphology between treatments.

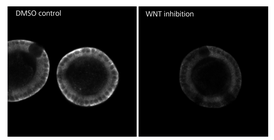


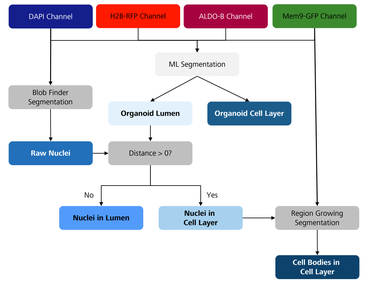
Fig 2B: **Aldolase B expression in organoids following control treatment (left) or Wnt inhibition (right).** Note the lower Aldolase B expression following Wnt inhibition.

Software Processing

The raw acquisition image data (one .czi file per organoid) was first batch imported into one Vision4D file (.sis). The raw image data set was then processed via image normalization in H2B-RFP, Mem9-GFP and DAPI channel to account for intensity differences between organoids and for signal intensity differences due to imaging depth.

Next, Machine Learning Segmentation was performed to segment the outer Organoid Cell Layer. The Organoid Lumen was next determined by filling inclusions in the Organoid Cell Layer segmentation.

Nuclei were segmented with the Blob Finder function from H2B-RFP and DAPI channels. Nuclei within the Organoid Cell Layer and the Organoid Lumen were separated into two object groups based on object distances to the Organoid Lumen. The Cell Bodies were segmented via Region Growing from Nuclei objects within the Organoid Cell Layer.



Validation

Next, the validity and quality of the different segmentations applied during the analysis was checked. Employing Machine Learning lead to superior segmentation results of the organoid cell layer compared to conventional threshold-based segmentation. It allowed discrimination of cells in the cell layer (included in the objects) and the lumen (excluded from the objects) based on complex image texture (Fig. 3A).

Cell nuclei were segmented with Blob Finder segmentation. This allowed high-quality separation of nuclei despite being densely packed in 3D and despite intensity variations. By setting up relationships with the organoid cell layer and lumen object, nuclei were then further separated into cell layer nuclei and luminal nuclei (Fig. 3B). Cell bodies were segmented by Region-Growing from cell layer nuclei. By object filtering, they are nicely restricted to the organoid cell layer (Fig. 3C).

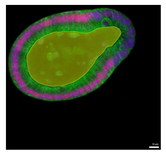


Fig 3A: **Organoid cell layer and lumen segmentation.** Cell layer overlay shown in green, lumen overlay in yellow.

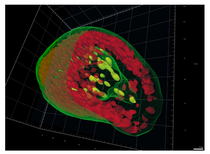


Fig 3B: **Nuclei in organoid cell layer and lumen (3D view).** Cell layer nuclei shown in red, luminal nuclei shown in yellow.

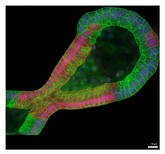


Fig 3C: **Cell bodies in organoid cell layer.** Cell layer nuclei shown in red, cell layer cell bodies shown in green.

Results

1. Size and Roundness of organoids

We observed in the initial overview image (Fig. 2A) that control-treated organoids formed more amorph shapes while organoids treated with Wnt inhibitor remained spherical. Vision4D offers several morphological parameters to analyze such observations. Fig. 4D shows a significant drop of "Roundness" in control-treated samples, hence, Wnt inhibition indeed interferes with the formation of amorph organoid shapes.

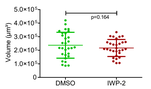


Fig. 4A: **The volume of the full organoids.** Single data points, mean and standard deviation depicted. p-value from statistical t-test is shown

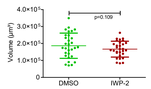


Fig. 4B: **The volume of the organoid cell layers**. Single data points, mean and standard deviation depicted. p-value from statistical t-test is shown

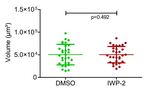


Fig. 4C: **The volume of the organoid inner lumen**. Single data points, mean and standard deviation depicted. p-value from statistical t-test is shown

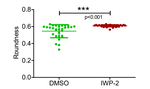


Fig. 4D: **The Roundness of full organoids**. Single data points, mean and standard deviation depicted. p-value from statistical t-test is shown

2. Cell numbers in different organoid compartments

For all three groups there is significantly increased cell numbers for control-treated organoids compared to organoids exposed to Wnt inhibition ($p<0.05$ each in statistical t-tests). This again indicates that Wnt inhibition interferes with proper organoid outgrowth. For organoid volumes, the spread of values (the standard deviation) is considerable. Analysis would therefore likely benefit from analyzing larger sample sizes.

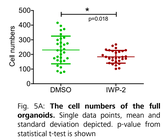


Fig. 5A: **The cell numbers of the full organoids.** Single data points, mean and standard deviation depicted. p-value from statistical t-test is shown

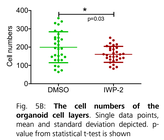


Fig. 5B: **The cell numbers of the organoid cell layers**. Single data points, mean and standard deviation depicted. p-value from statistical t-test is shown

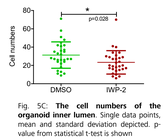


Fig. 5C: **The cell numbers of the organoid inner lumen**. Single data points, mean and standard deviation depicted. p-value from statistical t-test is shown

3. Aldolase B expression as a marker for enterocyte differentiation

Aldolase B is a marker for enterocyte differentiation. We show here the sum of Aldolase B expression for the complete organoid (Fig. 6B) and the single-cell mean Aldolase B intensities (Fig. 6C). In both cases, there is a significant increase in organoids that were mock-treated compared to organoids treated with Wnt inhibitor.

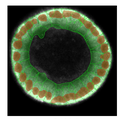


Fig. 6A: **Localization of Aldolase B expression in the organoids.** Aldolase B expression (gray) is localized to the entire cell bodies (green) rather than the nuclei (red).

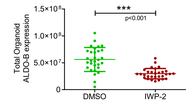


Fig. 6B: **Total organoid Aldolase B expression**. Single data points, mean and standard deviation depicted. p-value from statistical t-test is shown

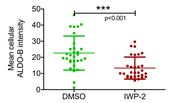


Fig. 6C: **Average cellular mean Aldolase B intensity** Single data points, mean and standard deviation depicted. p-value from statistical t-test is shown

4. Determining Aldolase B-positive cells as an alternative read-out

For the final section, the analysis strategy was to stratify cells into Aldoloase B-positive and –negative groups using a threshold and then determine the fraction of these cells within an organoid. Applying this threshold generates positive and negative cells that match well with the visual impression of Aldolase B distribution in the example cross-section (Fig. 7A). Fig. 7B shows the bimodal distribution of Aldolase B intensity over all cells in the data set. Results are shown as total positive cells per organoid (Fig. 7C) and as the percentage of positive cells per organoid (Fig. 7D). Again, control-treated organoids had significantly more Aldolase B-positive cells, indicating better organoid maturation.

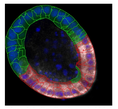


Fig. 7A: **Bimodal distribution of Aldolase B expression (grey) throughout the organoid.** Thresholding allows for good separation into positive (red) and negative (green) cells.

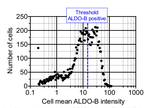


Fig. 7B: **Mean Aldolase B intensity distribution of single cells**. Note the bimodal distribution curve and the threshold set between the two maxima.

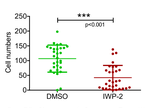


Fig. 7C: **Number of Aldolase B-positive cells per organoid**. Single data points, mean and standard deviation depicted. p-value from statistical t-test is shown.

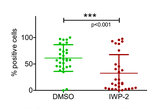


Fig. 7D: **Percentage of Aldolase B-positive cells per organoid**. Single data points, mean and standard deviation depicted. p-value from statistical t-test is shown.

Conclusions

In this application story we have been trying to showcase how to approach an organoid study employing a **ZEISS Celldiscoverer 7** and **arivis Vision4D** for image analysis.

Organoids are analytically demanding for their rather complex tissue architecture (3D data sets consisting of an outer cell layer and an inner lumen) and for the multitude of different read-outs that may be applied (organoid volume and shape, cell numbers and single-cell expression analysis of tissue differentiation markers). We have highlighted how all this can be accomplished with the built-in functionality of Vision4D.

In this experiment we found several read-outs that could highlight the role of Wnt signaling in intestinal organogenesis. We could measure, that organoids with inhibited Wnt signaling would have decrease organoid volumes, more immature spherical shape, less cell numbers and less Aldolase B expression. Aldolase B as a marker for enterocyte differentiation, was reduced both in terms of total expression levels and in terms of Aldolase B-positive cell fractions.

It should be noted that only a small set of 30 organoids per sample was analyzed, for the sake of demonstrating image analysis with our software. This certainly doesn't meet the high standards that what would be required for a professional study and statistically relevant conclusions. We still believe that having this kind of "real-world" use case motivates you to learn image analysis strategies to apply them to your own data.

References

1. Merenda A, Fenderico N, Maurice MM. Wnt Signaling in 3D: Recent Advances in the Applications of Intestinal Organoids. Trends Cell Biol. 2020 Jan;30(1):60-73. doi: 10.1016/j.tcb.2019.10.003. Epub 2019 Nov 9. PMID: 31718893. https://www.cell.com/trends/cell-biology/fulltext/S0962-8924(19)30166-7#%20

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