Universiteit Leiden
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Opleiding Bioinformatica

Optimizing the segmentation of zebrafish imaged with optical projection tomography

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BACHELOR THESIS

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21/08/2023

Abstract

The optical projection tomography (OPT) microscope method is becoming more interesting to use for imaging samples that fall between the ranges of confocal laser scanning microscopy (CLSM), which images tissues with a reach from micrometer to millimeter, and magnetic resonance imaging (MRI), a method for imaging large organisms starting from 1 centimeter. One of the most important models used in research is the zebrafish model which falls right in the range so that the entire organism can be imaged at once. Before experimentation, the zebrafish samples are prepared and imaged using the OPT method. This thesis focuses on visualizing the zebra fish and designing a workflow to be able to calculate the volume of the sample after being reconstructed and segmented with the help of the program Amira. It also investigates the difference in the volume after being treated. This was determined to not have an influence on the volume of the sample. The pipeline that was developed seems to have a similar effect to manually segmenting the model.

Contents

1 Introduction

One of the most fundamental tools for biological discovery is optical microscopy which has been used for the last three centuries. Unfortunately, in vivo, tissue has tested the limits due to light scattering. Thus, the most that can be done is a superficial investigation, including the more modern methods of confocal or multiphoton. Recent advances in optical imaging allow for imaging to be done at depths and resolutions unprecedented for optical methods [Ntz10].

1.1 The use

When working with a zebrafish a microscope needs to be used. Because of the size of the sample (having a length between 3.5 to 12 mm) [SH14], an Optical Projection Tomography (OPT) microscope is used. Besides the range of the microscope being good for zebrafish and other small organisms, it carries other advantages. The time spent on preparing and imaging a sample is also relatively short. This makes for a more efficient workflow during a study.

What has already been established is the protocol for the preparation of a sample, specifically a zebrafish, the embedding of the sample, and the imaging of a sample. The processing of the image of the sample misses a pipeline. This thesis is part of research to calculate the volume of the zebrafish sample.

The zebrafish used for this thesis do not have neuroblastoma but do have control injections. This thesis is part of a larger research to see the influence of treatment on neuroblastoma. It focuses on the development of the size of the zebrafish and of the neuroblastoma. To contribute to this I made a pipeline to calculate the volume of the zebrafish after OPT imaging.

For this project, I used the program Amira to make a pipeline to convert the reconstructed images from the OPT microscope into 3D models. After making a pipeline I also labeled the model by hand.

1.2 Research Question

The goal of this thesis is to answer the following research question: How effective is a pipeline made using Amira in calculating the volume of a zebrafish imaged with an OPT microscope? By making a pipeline consisting of several operations to make a visualization of the zebrafish I hope to answer this question.

1.3 Thesis overview

This chapter 1 contains the introduction. Section 2 gives the background on the relevant topics and previous research knowledge. The methods used in this thesis project are explained in section 3. The results are shown in section 4. Section 5 concludes the found results and section 6 discusses this thesis and plans for further work.

2 Background

2.1 Zebrafish

The zebrafish (Danio rerio), especially the zebrafish larva, get used more and more in drug discovery and early drug development. That is because the zebrafish model is very popular in biomedical research. It forms a link between in vitro experiments and in vivo studies in mammalian species. The zebrafish is an easy target to genetically modify to develop disease models because of its external fertilization, its large litter size, the size of the embryos and larvae, and the fast rate with which the larvae grow, which is ideal for high-throughput experiments.

Zebrafish are optically transparent during early embryonic and larval stages, so it is easy to image anatomical and certain physiological developments. Because there aren't any invasions, the fish can stay alive which has the benefit that effects can be observed by microscopy over time in a single subject [vW20].

The zebrafish is a specimen that is typically suitable for this type of imaging because its size fits in the range of the OPT microscope. Zebrafish can be easily embedded in large-scale projects as sufficient amounts of samples can be made available in a reasonable time. In experimental setups, we are highly interested in the phenotype and the gene expression of the phenotype in a zebrafish sample.

2.1.1 Related Work

Multiple articles have been published since 2000 using zebrafish as a cancer model. It was discovered that Zebrafish develop almost any tumor type known from humans. [HB19].

Genetics has proven to be a very powerful complementary approach to embryological studies, as genetic screens in zebrafish mutants have identified over 50 genes that are necessary for normal development [SBD00]. Results with zebrafish models have been positive enough to warrant their own niche in cancer research, complementing existing models with their experimental advantages. Examples of these are imaging of tumor progression in living fish, treating the tumors, and screening possibilities for genetic enhancers and suppressors [TL13].

Besides cancer there have been other ways to make models of zebrafish such as using CRISPR [CDDT18] and next-generation sequencing technology. The molecular mechanisms of human genetic diseases that are studied can be researched faster. These studies are fundamental for the future of precision medicine. Recently a study used zebrafish disease models for biomedical research in developmental disorders, mental disorders, and metabolic diseases[CCL+21].

2.2 OPT

Looking at figure 1 there are multiple microscope techniques presented with their corresponding range. With respect to CLSM, we are confronted with a limitation of the size of the specimen for whole-mount imaging. With MRI the strength of the magnetic field determines the resolution that can be obtained for whole-mount imaging. The OPT technique is a microscope method that can

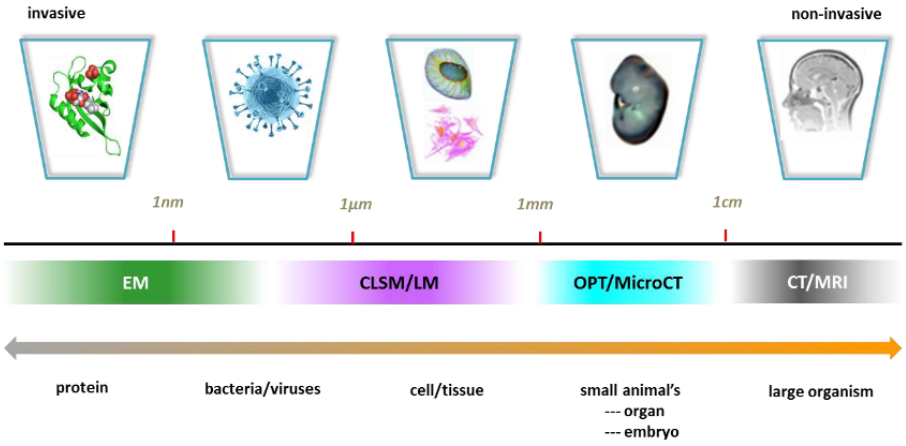

Figure 1: Overview of the scale and the microscope used for the range. The corresponding size of the sample is shown on the top [Tan20].

visualize gene expression or specific staining using bright-field and fluorescence channels, imaging the specimen as a whole. Because of this, it adds an important range of scale [Sha04]. It allows for the acquisition of high-resolution full-body images of animal or plant tissues as well as organs or even organisms and has been studied for the capability of imaging with good spatial resolution and contrast. After imaging it is able to make a reconstruction of the tomogram made from the sample.

The OPT can be advantageous over other available methods. When using OPT to image developmental processes, there is a possibility to monitor gene expression and anatomical alterations. The OPT microscope can use fluorescence and absorption as contrast[RBM+11]. The flowchart of the microscope is depicted in figure 2. In table 1 the important knobs of the OPT are visualized.

2.2.1 Related Work

Research regarding the OPT microscope and its potential has been going on since it was first developed [SAP+02]. There has been a lot of research with respect to improving the reliability and quality of this form of imaging. This research was continued, focusing on the application of OPT in biomedical research, dealing with the design and implementation of algorithms and computational strategies to deal with data, and images that are acquired with an OPT microscope [Tan20].

2.3 Amira

Thermo Scientific Amira Software is a software that can be used for visualizing, analyzing, and understanding different subjects related to life science. The data can be made using Optical and Electron Microscopy, CT, MRI, and other imaging techniques. It can visualize data related to structural and cellular biology and also tissues. From any 3D image data, Amira Software can make data visualizations but can also process and analyze the resulting model [lif]. The main focus of Amira is 3-D reconstruction and quantification of data[ASTM07]. Amira has the ability to make a workflow to combine segmentation and 3D reconstruction tools. It is also customizable,

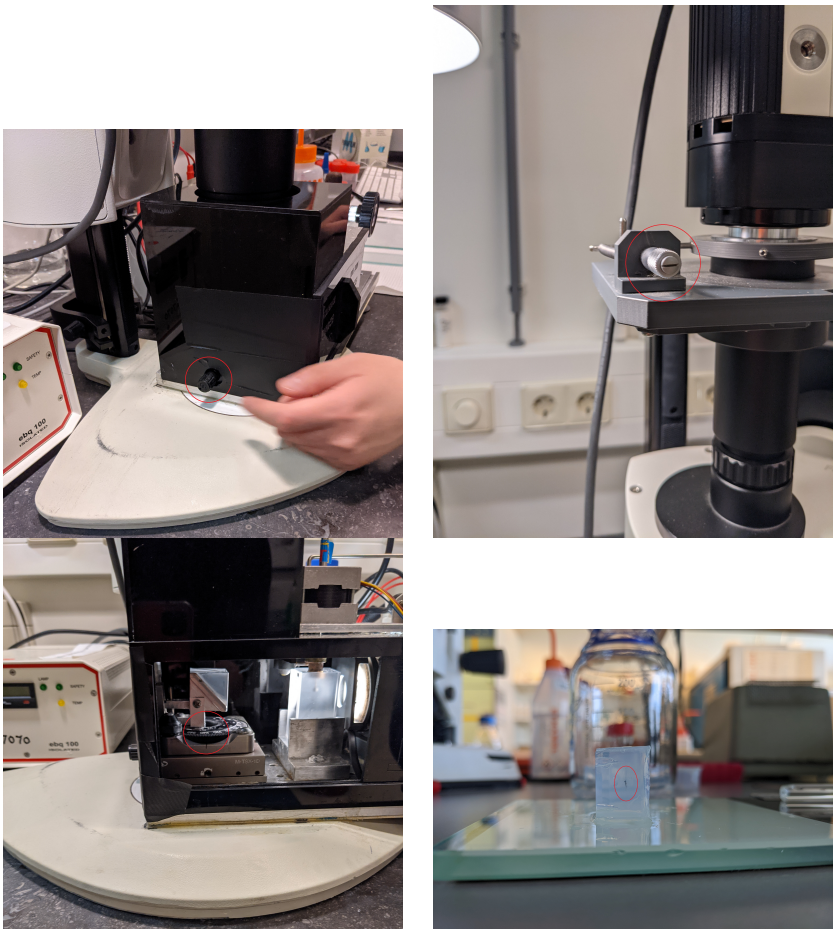

Table 1: The prism rotation knob is visualized in the upper left picture, the upper right picture shows the knob with which you can control the camera rotation. The lower left picture shows us the knob for the prism tilt and the lower right picture shows an embedded zebrafish almost ready for imaging.

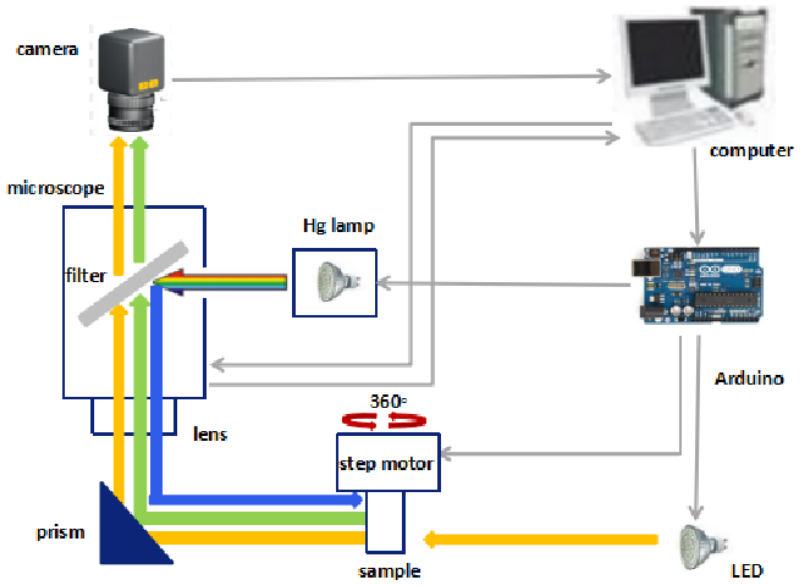

Figure 2: The diagram of an OPT imaging system. The path for the bright field channel is illustrated in yellow. [Tan20]

offering multiple coloring options for different tissues or organelles. There is a wide range of compatible import and export formats and simple workflows with either manual or semi-automated segmentation. What makes Amira truly unique is the option to incorporate MATLAB and Python, making it possible to apply deep-learning algorithms in the workflow. This variety and flexibility gives Amira advantages over other software, such as ImageJ [GLVK+21].

3 Methods

The methods used in this thesis can be divided into 2 components, those that were used to acquire the data and those that were than used to process the data. The workflow of the entire project can be seen in figure 3.

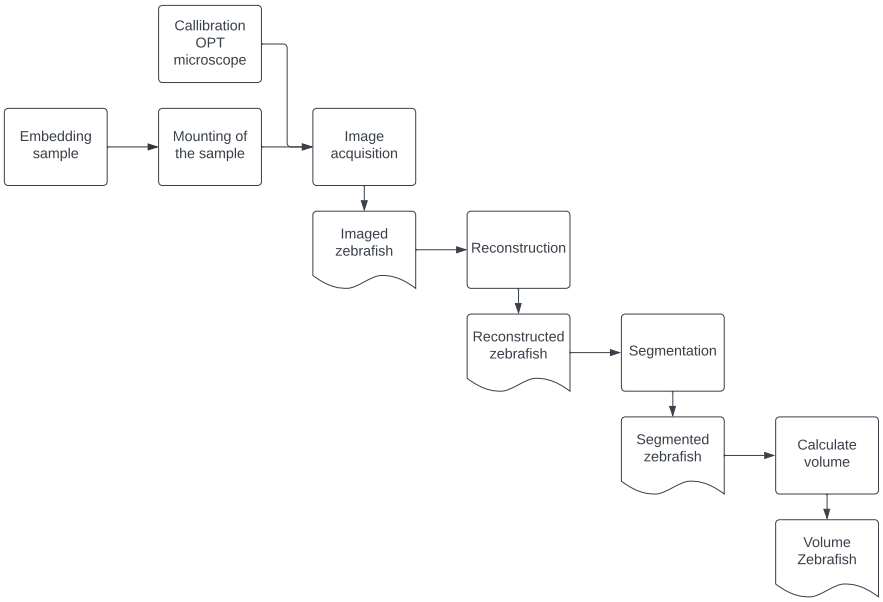

Figure 3: A workflow diagram for the methods used

3.1 Acquiring the data

A large part of this research revolves around getting the data to be able to construct the pipeline in Amira. To acquire the data the zebrafish samples needed to be prepared. The specific protocols were tuned to the specimens that were used, zebrafish larvae.

3.1.1 Embedding

When making use of an OPT microscope the specimen is fixed for imaging and is suspended in an agar gel cylinder that can be rotated. This step is the most time-consuming process in the OPT imaging workflow. After optimizing this process in other research, it still only allows the preparation of only a few samples per day. From proper OPT sample preparation and imaging, we acquire the tomogram data that is, in fact, a collection of axial 2D images [Tan20].

To prepare the zebrafish for imaging the protocol described in appendix A was used. The agar can be reused a couple of times but it is important to check the purity of the agar before every use. When making molds, the agar would need to sit overnight in the fridge to set. Because of this, it is necessary to plan ahead when using the microscope. When experimenting with embedding it was

noted that only 6 molds could be done simultaneously. This is because the agar is beginning to set the moment it is cooling down. When trying to mold more than 6 the time needed to position the specimen is longer than the time it takes for the agar to set and with the specimen in the agar, it is not a possibility to reheat the agar.

When demolding the specimen it is important to use a sharp blade to cut the casing. The best way to do this is by cutting the mold in all corners, with the direction faced lengthwise. This way you can cut along the agar instead of going into the agar, this will prevent damage to the specimen. After demolding is complete, it is necessary to cut out a cylinder due to rotating in the OPT microscope. When cutting out the cylinder a light-coloured workstation will give good contrast to the dark specimen in the clear agar. After these steps, the specimen is ready for imaging.

3.1.2 Calibration

Before being able to image the samples the microscope needs to be calibrated using the protocol described in appendix C. Every day the microscope would need to be calibrated before use. This could take up to four hours to complete. This practice ensures that when you are imaging the specimen is within the field of the camera at all times. This is also when you start deciding the magnification used to later image the specimen. In figure 4 you can see the OPT software when calibrating the microscope.

3.1.3 Imaging

After acquiring the agar cylinder it needs to be imaged from multiple angles to make an tomogram that can be used for later reconstruction. The OPT microscope uses a step motor and magnets to attach the sample as described in appendix B. When the sample is placed correctly we start with acquiring the image by using appendix D.

When imaging a new sample there are a lot of perimeters that can be adjusted to influence the end result. When imaging it was decided to stick to Brightfield as there would be no added benefits from using any filters. When deciding the number of angles that would be photographed there did not seem to be any benefit to decreasing the amount available. This is why every sample was imaged from 400 angles resulting in 400 pictures for every sample. When experimenting there also was the perimeter which decided the exposure time. After trying different settings there did not seem to be any big difference due to the fact that no filter or fluorescence was used. It was then decided to keep the exposure time at the default.

3.2 Processing the data

In order to visualize a zebrafish in 3D or further do quantitative analysis on it, the bright-field tomogram from the OPT imaging system is used. This tomogram consists of the 400 images in our OPT setup. The tomogram is then put into the reconstruction algorithm.

For volume region quantification in zebrafish annotation is required and is obtained based on segmentation of raw 3D image and subsequently visualized using Amira. There are two method

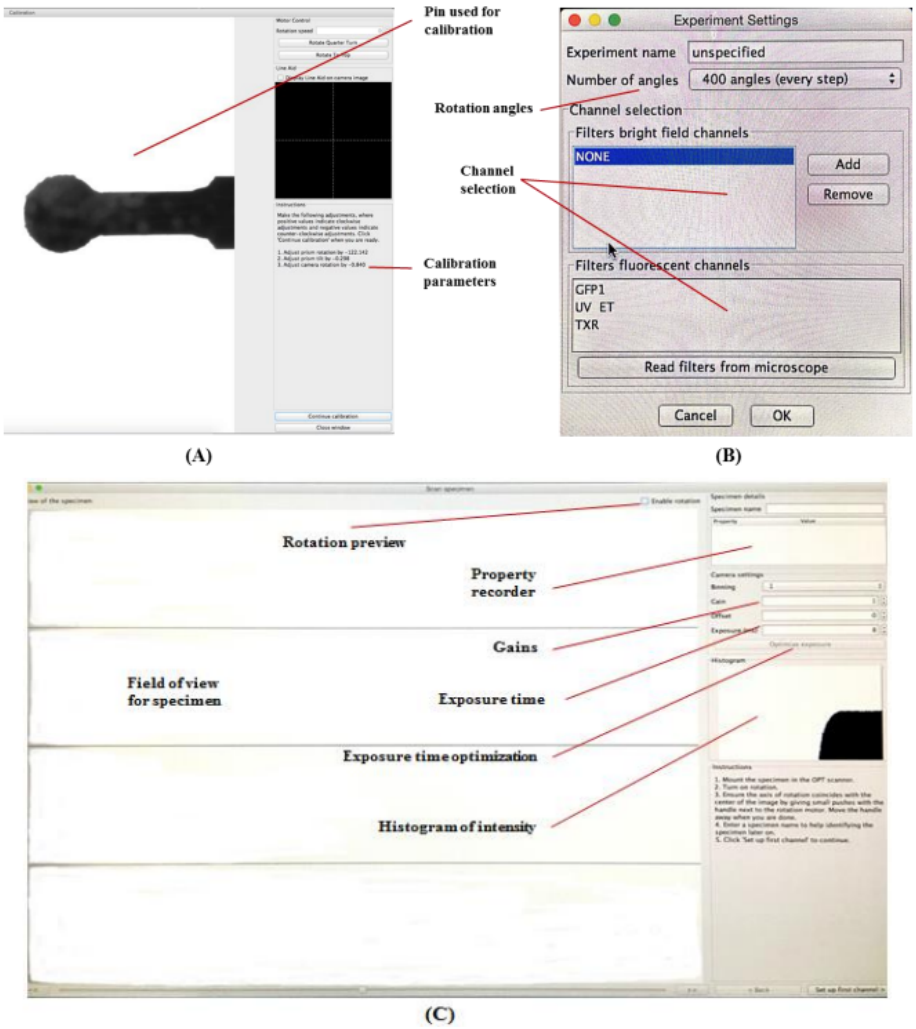

Figure 4: OPT imaging software. (A) The calibration user interface. (B) GUI for the experimental settings. (C) GUI for the bright-field imaging [Tan20].

used for determining the volume to check if the pipeline holds up to the volume calculated when manually labeling the data.

3.2.1 Reconstruction

By using a reconstruction algorithm there is a reduced amount of noise when processing the image further. The software is already installed on the OPT computer and can be used immediately after imaging the specimen. When reconstructing it is important to subtract the background image. This way any artifacts in the background will not interfere with the reconstruction of the sample.

When uploading the images into the reconstruction software it is possible to resize the image. This helps to make the process faster and also more compact. Due to this, the reconstructed files are all named after the size selected during the reconstruction process.

3.2.2 Pipeline

After looking into the possibilities provided by Amira A few modules and filters seemed promising but after looking into the filters they did not work the way that was expected. Due to this, it was decided that a pipeline would best fulfill the requirements to visualize and therefore quantify the volume of the zebrafish. The first method used is the pipeline in which the image is put through different modules with the purpose of optimizing the image and getting rid of the noise. The pipeline is pictured in figures 5 and 6 and will now be elaborated on.

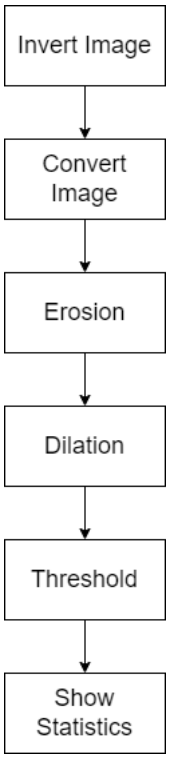

Figure 5: The pipeline established in Amira

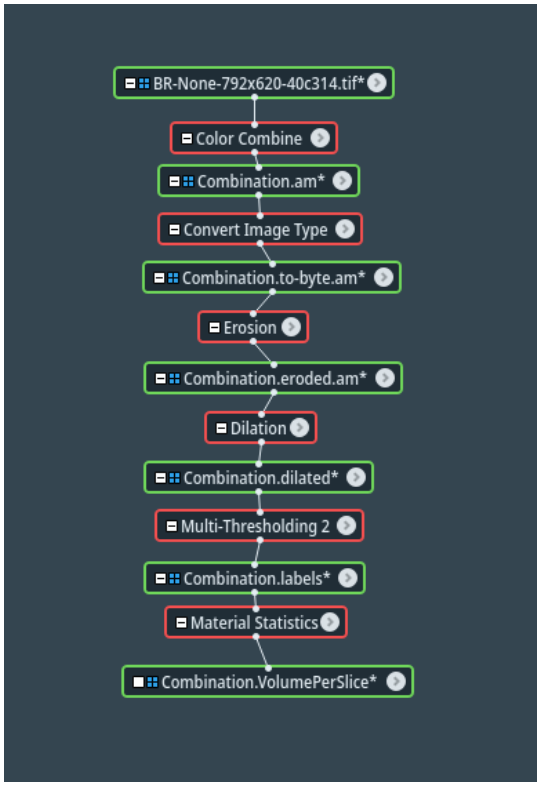

Figure 6: A screenshot of the modules used in order to acquire the measurements

Color Combine - This module is used to invert the image. When loading in the image it is a white background with the object in black. This is due to the imaging acquisition process where we shine white light onto the zebra fish which absorbs it. The background does not absorb any light so it gets reflected back to the camera which presents itself as white. Further along in the process, it is necessary to have this inverted to differentiate the foreground from the background. That is why it is important that the object on which we focus white is.

Convert Image Type - After using the color combine module the image is converted to a format that can't be used by other modules. That is because the number of bits per pixel must be 8 or 16. Due to the previous operation on the file, this was no longer the case. This is why it needs to be converted to a standard measure using this module. This results in an image that you can

manipulate in other modules.

Erosion- This is part 1 of a standard filter. In the image, there is noise in the form of bubbles. In an effort to get rid of the smaller ones, we make use of the method of eroding and dilating the image. This module erodes the image by 3px.

Dilation- This is the standard follow-up after using the erosion module and is used to dilate the image. The image needs to be dilated using the same factor as the erosion so the image gets dilated by 3px. This completes part 2 of the filter.

Multi-Thresholding - This module is the only thresholding module when not using extra packages. It was used to separate the foreground from the background by setting the threshold, which was personalized to every sample. After this was done you can calculate the volume of the image.

Material Statistics - The Material Statistics module was only used to visualize the results after using the pipeline. This module had a pop up window which displays the amount of voxels in the interior.

3.2.3 Manual

When proccessing the image manually we make use of the labels acquired by the pipeline and further elaborate them by using the segmentation function made available in Amira. By scrolling through the individual slices it was possible to add and subtract from the interior and exterior when interference in the form of bubbles presented themselves in the sample. The dashboard can be seen in figure 7. In this figure you can also see the artifects that are seen as volume but that are outside of the model of the zebra fish.

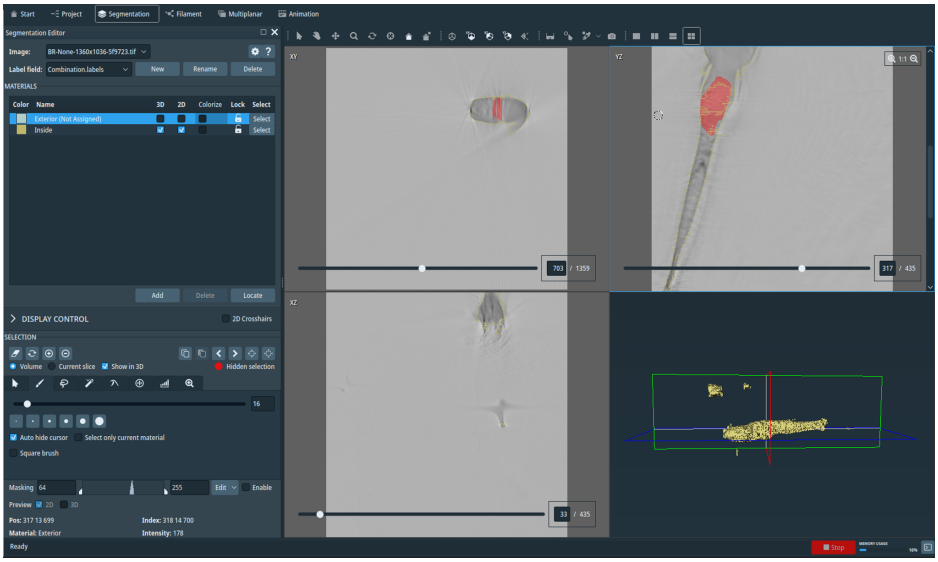

Figure 7: A screenshot of the segmentation used in order to acquire the data

4 Results

For this study 24 samples were used, half of them were injected with Polyvinylpyrrolidone (PVP), and the other half were injected with mWasabi (WA). The PVP samples are a control group for the WA samples. WA is a green fluorescent protein, but because of the imaging in brightfield there are no functional differences between the 2 groups.

The samples have been imaged and reconstructed. With Amira they were segmented using the pipeline method explained in section 3.2.2 and as a control manual segmentation was also performed. After using the pipeline you get a result as seen in figure 8, a clear outline of the zebrafish, but also containing bubbles outside. After manually adjusting the segmentation the result is seen in figure 9, no bubbles inside or outside the zebrafish. The results can be seen in table 2 for the samples injected with PVP and in table 3 for the samples injected with WA. The first column represents the name of the sample. The second column gives the number of voxels that were measured using the pipeline method and the third column uses the manual method. The fourth column gives the magnification used when imaging the sample. The fifth column gives us the difference in volume between the two methods that were used.

4.1 Pvp injection

In figure 10 the results for the PVP injection are visualized with the biggest difference in volume measured between the two methods seen in sample 1.

4.2 mWasabi injection

In figure 11 the results for the WA injection are visualized with the biggest difference in volume measured between the two methods seen in sample 6. In figure 12 all the results can be seen and give one outlier in sample 6 with the WA injection.

Sample (Polyvinylpyrrolidone)	Pipeline in voxels	Manual in voxels	Magnification	Difference in voxels
452x372-039a9a	2137909	2439143	12.1	301234
508x632-dc6b9a	2116368	2215508	12.1	99140
612x508-f33d78	2222864	2160550	12.1	62314
688x812-fe1719	1818145	1780455	12.1	37690
468x652-8aeb8a	1763472	1726262	12.1	37210
560x520-0579ee	2498473	2276993	12.1	221480
588x528-361861	2084724	2116321	11.6	31597
484x748-405115	2192813	2113733	11.6	79080
684x644-90216d	2163892	2091836	11.6	72056
508x432-f24c74	2102714	2039285	11.6	63429
556x684-721e49	2202999	2267322	11.6	64323
456x524-42974d	2073926	2092652	11.6	18730

Table 2: The results from samples with the Polyvinylpyrrolidone injection

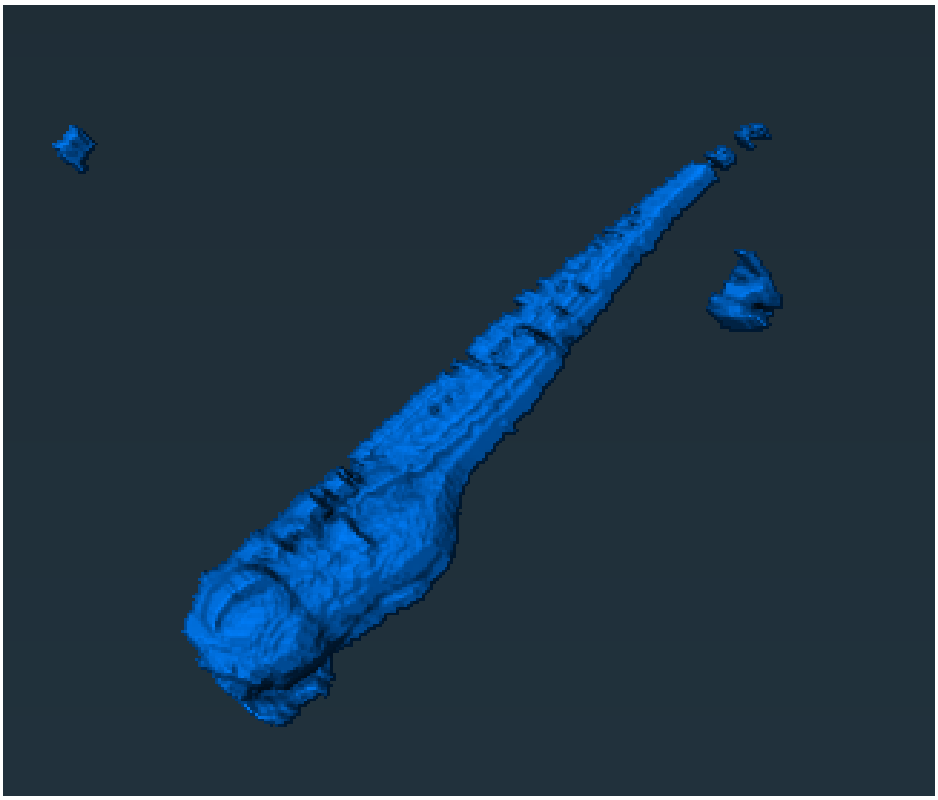

Figure 8: The resulting segmentation after using the pipeline

Sample (mWasabi)	Pipeline in voxels	Manual in voxels	Magnification	Difference(μm^3)
496x944-07fdba	2107573	2000451	11.5	107122
692x752-8fe2ee	1957536	1854169	11.5	103367
620x834-dd3fee	2075399	2261709	11.5	186310
627x660-0f595f	1848891	1869269	11.5	20378
608x792-5b97e1	1596778	1764811	11.5	168033
788x752-c6270f	1446363	1228832	11.5	217531
668x660-ef2170	2185732	2107573	11.5	78159
740x860-a4b3aa	2084621	2195725	11.5	141104
648x688-019d11	1937264	1860572	11.5	76692
600x428-b73300	1803982	1850285	11.5	46303
708x652-105c5f	2019845	2063924	11.6	44079
636x508-8434ca	1964502	1942943	11.6	21559

Table 3: The results from samples with the mWasabi injection

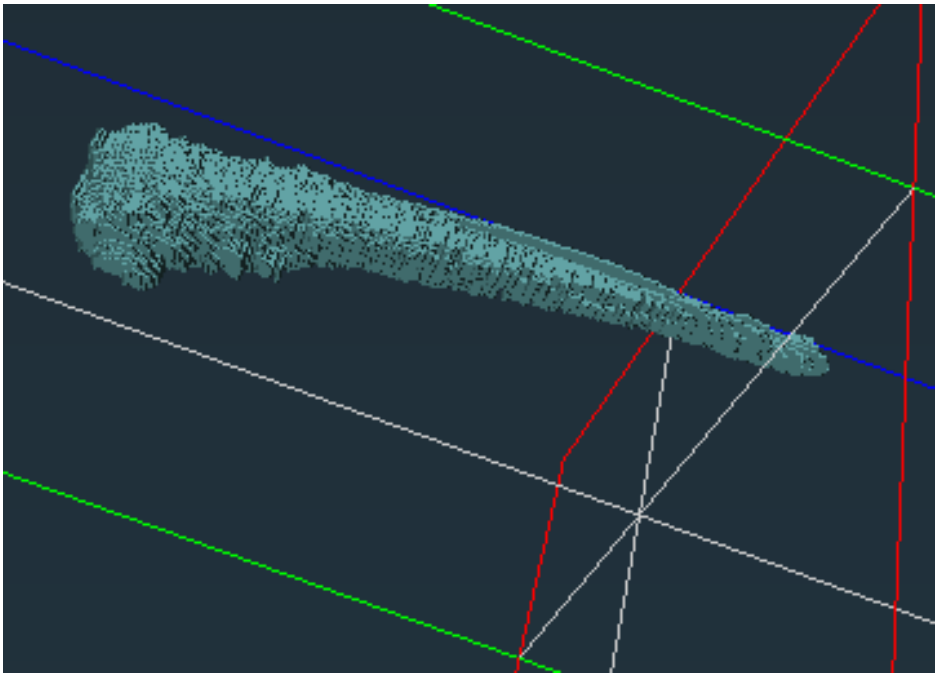

Figure 9: The resulting segmentation after manually adjusting the segmentation

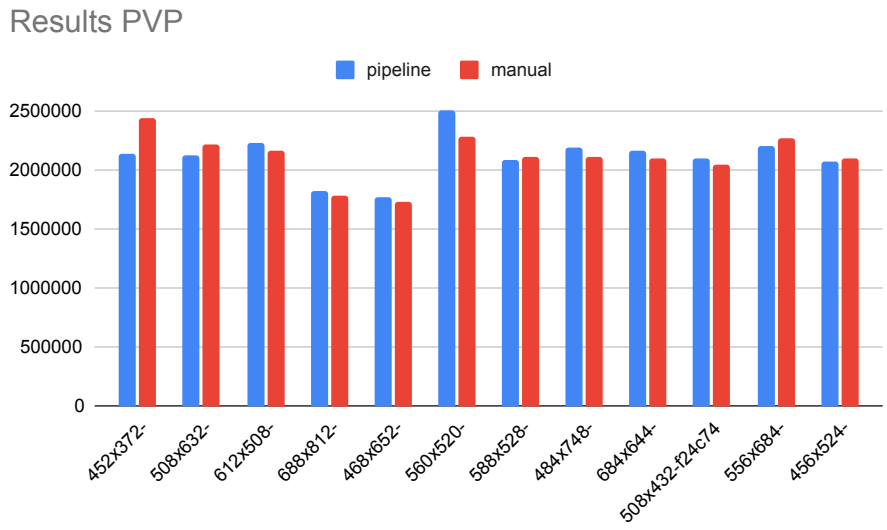

Figure 10: The results from samples with the Polyvinylpyrrolidone injection volume using the pipeline method versus manually segmenting the model

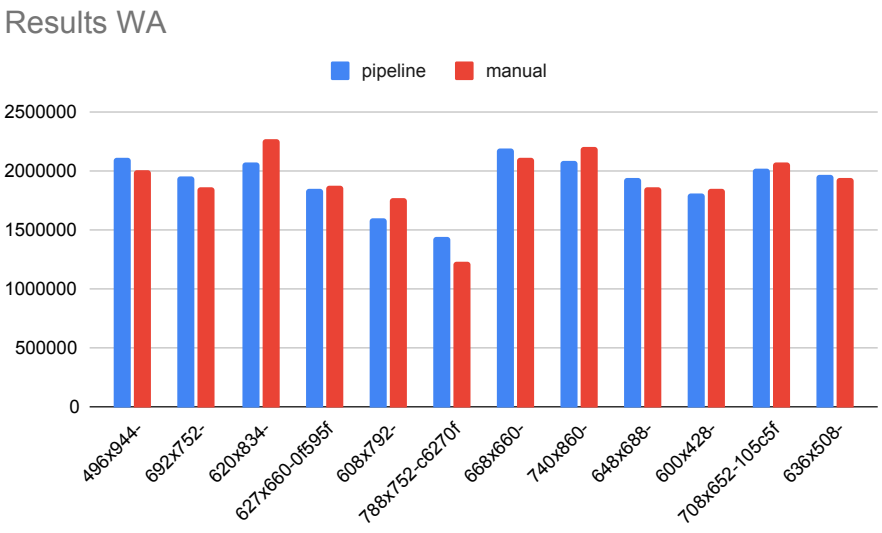

Figure 11: The results from samples with the mWasabi injection volume using the pipeline method versus manually segmenting the model

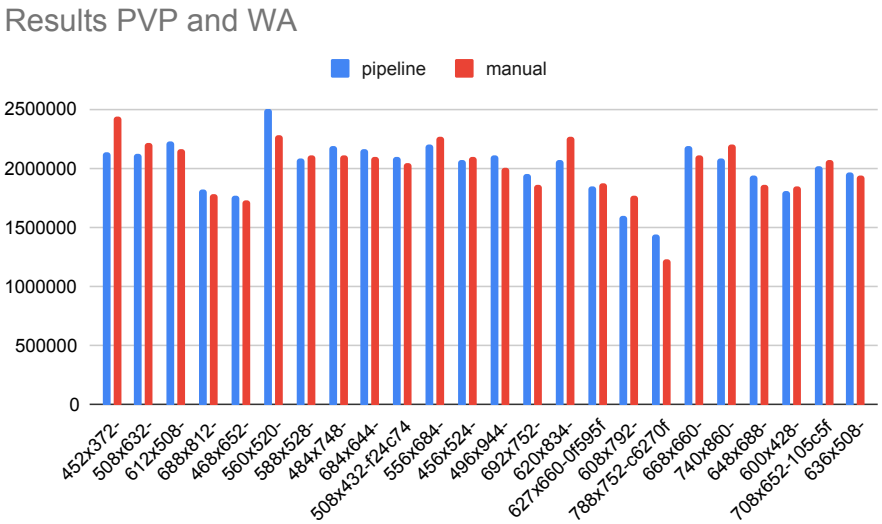

Figure 12: The results from the WA and PVP volumes set out against each other in a graph

5 Conclusions

In conclusion, 24 zebrafish were prepared and observed during the study, 12 were treated with PVP, and 12 were treated with WA. To answer the research question: the made pipeline is very effective to calculate the volume of the zebrafish and the biggest difference that was recorded was 301 234 voxels on a volume of 2 439 143 voxels. The smallest difference was 18730 on a volume of 2 092 652. The pipeline made for this study gives compatible results when calculating the volume using manual segmentation but has still room for improvement if the difference between samples can be that high. The differences between the samples treated with PVP and with WA are not noticeable.

5.1 Comparison

When reconstructing the zebrafish with the pipeline bubbles can appear outside the sample due to contamination and inside the model, small gaps can occur. This affects the end results when calculating the volume. When manually reconstructing the zebrafish this problem does not occur so that gives a clearer calculation. The time spent manually modeling the fish takes around an hour per sample to go through all the frames in the image stack. The pipeline takes around 6 minutes per sample.

6 Discussion and Further Research

6.1 Preperation

When embedding samples we reuse the scraps of agar gel for the next embedding. After multiple uses the gel gets contaminated which causes bubbles to appear in the reconstruction. This is a point that can be improved on by regularly checking the agar before and after use. Also making additional steps in the protocol such as using the vacuum chamber after heating the agar gel.

The differences between the results of the pipeline and the manual segmentation is up to 12%. A cause of this could be the bubbles that are caused when preparing and embedding the samples. These bubbles will then show up when using the OPT machine and in the zebrafish model when reconstructing in Amira. The bubbles that appear outside the zebrafish will count as 'foreground' volume, adding to the total volume. The bubbles inside the sample will count as 'background', taking away from the volume. The difference between the results of the pipeline method and the results of the manual method is the impact of the noise that the bubbles make on the model.

6.2 Treatment

There is no big difference between the samples treated with PVP and the samples treated with WA the injections are not meant to have any influence on the processes of the sample. In this research, no fluorescent imaging was used but for further research, it can be used to label the zebrafish which would justify the use of WA samples.

In this research, there was no observed difference in volume because the zebrafish were all the same sort. To control the functionality of the pipeline, other zebrafish samples with a variety of volumes should be tested. If the calculated volume from the pipeline is corresponding to the pipeline, we are able to verify the working of this project.

6.3 Furter Research

Further research needs to be done in optimizing the pipeline by getting rid of the bigger bubbles present in the image perhaps by using extensions in Amira which open up more modules for manipulating the image. A lot of the modules that were mentioned in the official Amira guide were only accessible after installing extensions but there seemed to be a few promising extensions for removing bubbles outside the zebrafish and filling gaps inside the zebrafish. These would need to be tested in future research.

Another possibility would be to use different modeling software to compare the time and quality for visualizing zebrafish.

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[vW20] Rob Christiaan van Wijk. *Translational pharmacokinetics-pharmacodynamics in zebrafish: integration of experimental and computational methods*. PhD thesis, Leiden University, 2020.

A Embedding the sample

1. Heat up 1% low melting point (LMP) agar in the microwave. Be careful it does not overheat.
2. Prepare the embedding mold with the flat bottom on the freezer plate.
3. Fill up $\frac{3}{4}$ of the embedding mold with 1% LMP agar.
4. Let the 1% LMP agar set until it is partly liquid and partly solid.
5. Meanwhile, cut the edge of a plastic pipette with a blade.
6. Transfer one zebrafish with the plastic pipette to the embedding mold that is filled with 1% LMP agar. Make sure the zebrafish stays put around the tip, otherwise it is possible the zebrafish gets stuck in the pipet.
7. Place the zebrafish in the middle of the embedding mold. We can manipulate its position with a toothpick.
8. Incubate the samples in the fridge for a minimum of two hours until the 1% LMP agar is solidified completely.
9. When the agar is solid:
   (a) Remove the embedding mold if necessary.
   (b) Cut the sample with a circular apple cutter. Make sure that the sample is in the center of the cut.
10. Store the samples in PBS.
11. Transfer the excess 1% LMP agar back in a flask for future use.

B Mounting of the sample

1. Dry the sample using a lens tissue paper.
2. Apply glue to the plastic cilinder and wait until it turns matte, then attach the specimen.
3. Let the glue harden (minimal time is 1 hour).

C Calibration of the OPT microscope

1. Turn on the microscope and the camera.
2. Mount the calibration pin to the magnet beneath the stepper motor.
3. Start the OPT software and open the calibration menu.
4. Zoom in until the image doesn't contain any edge of the lamp.

5. Adjust the position of the pin such that it stays within the image during rotation.
   (a) Make sure the top half of the pin is visible by adjusting the prism position.
   (b) Set rotation speed to 10 to let the pin rotate. If the pin does not stay inside the image, stop rotation, adjust the zoom and position of the pin. Repeat until the pin is positioned properly.
6. Adjust focus until the pin is in focus.
7. Calibrate the microscope according to the software. Once the microscope has been calibrated correctly, nothing should be changed anymore.
   (a) Set the prism rotation by turning the black knob next to the prism. The horizontal line shown in the software should be aligned with the dashed line. Press "Continue calibration". Repeat until prism rotation is within the bounds indicated by the software.
   (b) Set the prism tilt by turning the metal knob next to the prism. There is no visual feedback, so use small turns. Press "Continue calibration". Repeat until the prism tilt is within the bounds indicated by the software.
   (c) Set camera rotation by turning the metal knob next to the camera. Use the same process as the prism tilt to calibrate the microscope. NOTE: during calibration changing one value can influence another.

D Image acquisition

1. Start an experiment in the Experiment menu of the OPT software. The number of angels and filters can be chosen. For brightfield choose filter "None".
2. Start a new scan in the Experiment menu of the OPT software.
3. Mount the sample.
4. Name the specimen and add relevant information.
5. Press "Set up first channel".
6. If necessary, change parameters.
7. Press "Start scan".
8. After imaging, access the quality of the image and if needed, redo the scan (press "Re-scan"). Otherwise press "Save image".
9. If multiple channels were set up repeat steps 6-8.
10. Remove the sample when prompted and take background images.