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Shape analysis for phenotype characterisation from high-throughput imaging

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Chapter 3

Shape-based 3D Reconstruction and 3D Measurements

Based on:

- Y. Guo, W.J. Veneman, H.P. Spaink & F.J. Verbeek, “Silhouette-based 3D model for zebrafish high-throughput imaging” in IEEE Conference on Image Processing Theory, Tools and Applications, Orleans, France, 2015, pp. 403-408.
- Y. Guo, W.J. Veneman, H.P. Spaink & F.J. Verbeek, “Three-dimensional reconstruction and measurements of zebrafish larvae from high-throughput axial-view *in vivo* imaging,” Biomedical Optics Express, vol. 8, no. 5, pp. 2611-2634, 2017.

3. SHAPE-BASED 3D RECONSTRUCTION AND 3D MEASUREMENTS

This chapter addresses RQ 1 and RQ 3.

RQ 1: *To what extent is it possible to develop an MM-HTAI architecture for the zebrafish larvae?*

RQ 3: *To what extent is it possible to obtain precise 3D shape description and derive accurate 3D measurements that are statistically relevant for the zebrafish from the MM-HTAI architecture?*

Abstract – High-throughput imaging is applied to provide observations for accurate statements on phenomena in biology and this has been successfully applied in the domain of cells, i.e., cytomics. In the domain of whole organisms, we need to take the challenge to ensure that the imaging can be accomplished with a sufficient throughput and reproducibility. For vertebrate biology, zebrafish is a popular model system for high-throughput applications (see Section 3.1). The development of the Vertebrate Automated Screening Technology (VAST BioImager), a microscope mounted system, enables the application of zebrafish high-throughput screening. For the VAST BioImager, fluorescence and/or confocal microscopes are used. Quantitation of a specific signal as derived from a label in one fluorescent channel requires insight into the zebrafish volume to be able to normalise quantitation to volume units. However, from the setup of the VAST BioImager, a specimen volume cannot be straightforwardly derived.

We first present the multi-modal high-throughput axial-view imaging architecture by the employment of different types of microscopes based on the VAST BioImager (see Subsection 3.3.1). We then propose shape-based 3D reconstruction to produce 3D volumetric representations for zebrafish larvae using the axial-views (see Subsection 3.3.3 and 3.3.4). Volume and surface area are then derived from the 3D reconstruction to obtain the shape characteristics in high-throughput measurements. In addition, we develop a calibration and a validation of our methodology (see Subsection 3.3.5). In Section 3.4, from our experiments, we show that with a limited amount of views, accurate measurements of volume and surface area for zebrafish larvae can be obtained. We have applied the proposed method on a range of developmental stages in zebrafish and produced real metric references for the volume and surface area of each stage (see Subsection 3.4.4).

3.1 3D shape description for zebrafish

The application of high-throughput imaging is wide-spread in modern molecular genetics based biology. Imaging takes an important position in a variety of high-throughput applications and the image based applications are found on the cellular, tissue-culture and organismal level. High-throughput is a demanding process and as throughput is required, the optics setup needs to cover for sufficient image quality in further processing of the samples.

Zebrafish is a popular model system to study a broad range of biological phenomena. A lot of these phenomena are rooted in molecular genetics, i.e., developmental biology, toxicology, cancer, infectious diseases, and drug targeting. In an experimental setting zebrafish are easy to breed and per crossing about 200 eggs can be obtained. The early embryonic stages are easily studied through microscopy as well as easy to manipulate. In the past decade, through genetic engineering, a large amount of transgenic zebrafish lines incorporating Green Fluorescent Protein (GFP, and the like) as a reporter gene have become available. These lines, and the genes they represent, support the research in that the gene-expression from the transgenic line helps indicating specific events in space and time. The GFP-like reporter genes are studied through fluorescent and confocal microscopy and the fluorescence facilitates measurability of the expression so that a numerical representation of experiments can be obtained.

Zebrafish, augmented with the large amount of reporter lines, are quite suitable for high-throughput experiments given the number of embryos that can be obtained for experimentation. Furthermore, the genomics of zebrafish is close to human. Therefore, zebrafish is now massively used as a model in disease studies which are often performed in a high-throughput setting. Initially, these experiments were designed to such a way that the read-out was realised using 2D images and subsequent processing of these images. In this manner a kind of mid-throughput could be accomplished. The space and time features of these images could, de facto, be used efficiently. In order to scale experiments to true high-throughput, other solutions need be probed [7]. Moreover, spatial acuity is required to enable the 3D image analysis. Apparently, confocal laser scanning microscopy (CLSM) seems the best option. So, in addition to the microscope and automated acquisition protocols, devices are required to make it possible to do accurate and fast imaging, both 2D and 3D, on life specimens and, if possible, in a time-lapse fashion. A solution has been presented through the adaptation

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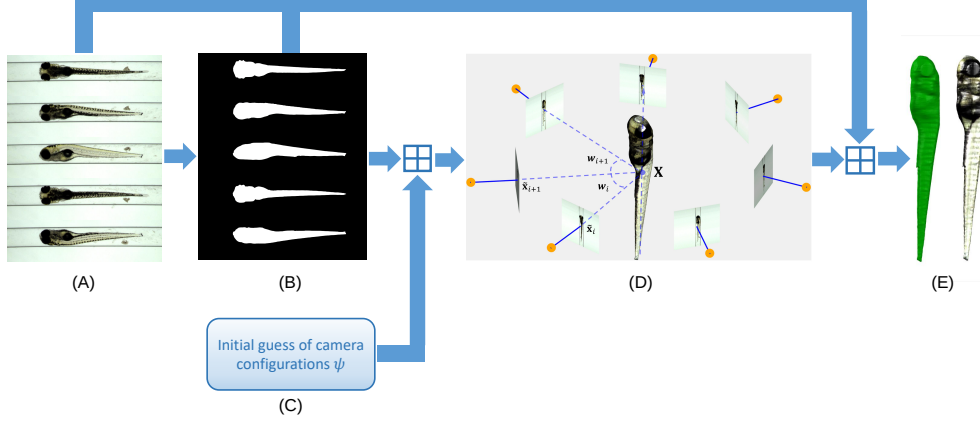


Figure 3.1: Flowchart of the 3D reconstruction and measurements of zebrafish larvae for the high-throughput axial-view imaging system. (A) Axial-view images of zebrafish as acquired from the VAST camera. (B) 2D shapes are obtained from the segmentation of axial views. (C) Initialisation of camera configurations estimated from the VAST BioImager. (D) Visualisation of parameterisation and calibration of the axial-view camera system. The bold dots represent centre of the camera lens; the dash lines represent the principal axis of the camera. (E) Reconstructed 3D zebrafish models with a volumetric representation shown on the left and a texture-mapping model on the right. The $\boxplus$ symbol indicates the integration of different computational modules. For further details see Section 3.3.

of screening hardware from the invertebrate field and making it suitable for the vertebrate field, more specifically for zebrafish; i.e., the Vertebrate Automated Screening Technology (VAST BioImager) [26]. The VAST BioImager extends a microscope, equipped with (epi-)fluorescence and/or confocal imaging options. Through a capillary system, zebrafish embryos/larvae are delivered, transported and kept in a fixed position for imaging. The capillary is mounted such that it can rotate while the specimen is kept in its fixed position. In this manner, images from different angles, i.e., axial-view images, can be acquired and analysed. Some typical examples of the axial views obtained by the VAST camera are depicted in Fig. 3.1 (A).

In order to obtain successful image analysis of zebrafish, accurate and precise shape measurements are important, especially for providing a reference of the overall size and shape. In fluorescence, only the specific shape characteristics from specific labels can be analysed. In contrast, bright-field images will give all information on shape and size if they present an overview of the specimen. Therefore, we investigate how the axial-view images can be employed to obtain precise

and accurate measurements on size and shape [40]. We first design a multi-modal high-throughput axial-view imaging (MM-HTAI) architecture based on the VAST system and light microscopy. This architecture can produce a full revolution of the specimen and acquire, with the VAST camera, a set of axial-view images. A potential of the MM-HTAI architecture is to produce detailed microscopic images both in bright-field and fluorescence. Subsequently, we will investigate how these images can be used to reconstruct a volume and obtain the size and shape measurements. These measurements then serve as a reference of size and shape of more specific features. A general overview of the proposed method is shown in Fig. 3.1. Given the microscope setup with the VAST BioImager, axial-view shapes of the zebrafish as shown in Fig.3.1 (B) are extracted from the original images as shown in Fig. 3.1 (A) and, henceforth, a 3D volume can be produced through combination of the shapes. The underlying idea of the 3D reconstruction from axial-views is similar to the problem of multi-view based 3D vision. To that end, a camera calibration model needs to be developed as framed in Fig. 3.1 (D). The 3D reconstruction can be accomplished once the segmentation and camera calibration are in place. An example of the 3D reconstructed zebrafish model is depicted in Fig. 3.1 (E). We need, however, to make sure that the result is a good representation of the shape; i.e., the measurements that we derive from the model can be considered correct. So, validation experiments and evaluation of the methodology on zebrafish larvae will be performed.

In order to clarify our method, the following issues are addressed in the next sections of this chapter. In Section 3.2, a number of 3D reconstruction methods are reviewed and the proposed method is motivated by the 3D reconstruction based on shapes, i.e., the binary masks obtained from segmentation of the object. In order to apply the method, an accurate estimation of the camera configuration is required. Therefore, we elaborate this issue for microscope optics and include some necessary optimisation steps for efficient computation in Section 3.3. In Section 3.4, we continue with a validation of the proposed method by introducing calibration beads that are used in an initial experiment testing whether our setup can produce accurate reconstructions. In addition, we test whether our measurement algorithms are sound. Subsequently, we have performed experiments on a large set of zebrafish larvae and shown successful applications of the proposed method for zebrafish larvae. The experiment deals with a range of developmental stages in zebrafish and the outcome of the measurements is interpreted. One of

the results is a graph of the distribution for volume and surface area of the zebrafish larvae. We explain how this distribution is derived from the experimental results. We present the major conclusions from this research, answer the RQ 1 and RQ 3, and extrapolate to future work in Section 3.5.

3.2 Background and method motivation

In multi-view 3D reconstruction methods, two major categories can be discerned. The first category is based on epipolar geometry and aims estimating the depth information by matching the corresponding points from correlated images of one identical scene using geometrical clues [67, 68]. The methods use texture mapping to define point similarity or disparity, but require pixel-wise correspondence. The second category concerns the multi-view 3D reconstruction by producing a volumetric representation of the object from a series of binary masks of the shape, which sometimes is referred to as the silhouette based 3D reconstruction.

For the 3D measurements that we wish to extract from the zebrafish, the 3D shape plays an important role. Our method should, thus, have an innate shape preserving ability. Due to the partial transparency of the zebrafish embryos, it is difficult to determine point correspondences from the object surface within the multi-view images. Therefore, the silhouette based 3D reconstruction method is more suitable for this type of data. In this chapter, a binary mask is derived from an axial-view image which is preferably defined as a shape. As of now, we thereby use, in the remainder of this paper, the term shape-based 3D reconstruction to indicate our reconstruction method.

In the silhouette based method, the initial idea takes into account the parallel projections derived from each individual shape [69] to generate a 3D intersection. Various 3D volumetric representations are then proposed. Hierarchical resolution voxels are used for an efficient construction of the 3D object [70]. The voxels that are populating the object surface are represented with a high resolution grid, while the voxels inside the object are represented by much coarser grid. Such data structure is commonly referred as an Octree. Due to the characteristics of the camera imaging system, cone-shaped projections have been derived from the camera configurations [31, 71]. The silhouette based 3D reconstruction methods have become more feasible through the introduction of the concept of the visual hull [72, 73, 74]. The space carving algorithm [75, 76], as conceptualised in the

space-carving theory, is an important implementation of this concept. In this method, based on the pinhole camera projection model, all the silhouettes are back-projected to the 3D space represented as a voxel-space. According to space carving theory [76], only the voxels visible to each of the silhouettes are preserved and used to reconstruct the final 3D model.

Other methods combine the information from texture mappings of the original images; one of which includes the surface reflectance to generate more natural 3D scene [77]. In recent approaches [78, 79, 80, 81], the 3D reconstruction is accomplished by searching for an optimal surface. The 3D surface is embedded in a higher dimensional space. Each voxel candidate included or excluded by the enclosed surface is assigned with a probability which indicates the possibility of the voxel candidate belonging to the object or the background. The probability is modelled by a multi-variate Gaussian kernel which is applied on the texture mappings interactively indicated by users. An energy function is subsequently defined by incorporating the total probability and the total surface area. Finally, the variational framework is taken to solve the optimisation problem. Recently, innovations [82, 83] were reported, which (1) are carefully formulated to preserve and refine the details across the projective rays on the object surface by introducing various constraints, and (2) achieve elaborate results on public datasets.

Our aim is to accomplish 3D measurements from our high-throughput imaging system. From the related work, we have taken our inspiration to further develop the multi-view based 3D reconstruction and introduce new ideas for the calibration of the optical setup in microscopy. Our point of departure is an existing opto-electronic configuration for high-throughput imaging to accomplish a reconstruction of zebrafish larvae. As a consequence, in order to be able to produce reconstructions from multi-view imaging, a calibration of the microscope imaging system needs be performed. To that end, a solution for the calibration problem is proposed using a revisited objective function in the optimisation. This function is defined as the voxel residual volume (VRV) maximization problem, which is a simplified formulation of the area coherence [84]. In terms of implementation, VRV is more efficient compared to the silhouette coherence [85]. In order to validate the proposed method, a large dataset of zebrafish larvae of different developmental stages is produced. For each of the instances in this dataset, a multi-view image set is acquired. From our method, 3D reconstructions are produced including the generation of dense surface points and triangulated meshes [86]. From the volumetric reconstructions and the meshes, volume and surface

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area can be computed. In addition, the method is applied to calibrated spheres in order to obtain metrical references. With a statistical analysis of the dataset, we have obtained a metrical reference for the most frequently used developmental stages of zebrafish in High-throughput imaging. This analysis will provide a baseline evaluation for future research in high-throughput imaging of zebrafish as well as a baseline for volume and surface estimations in zebrafish development providing reference values for a range of other features.

3.3 Materials and methods

Below we elaborate on the MM-HTAI system in Subsection 3.3.1. Our starting point is images that are acquired with the system; from these images, the shapes (silhouettes) need to be extracted using the method proposed in Chapter 2 which is also discerned in Subsection 3.3.2. We parameterise the camera model in Subsection 3.3.3, which is essential to the shape-based 3D reconstruction as elaborated in Subsection 3.3.4. For a good shape-based 3D reconstruction, the camera calibration should be operated and is addressed in Subsection 3.3.5.

3.3.1 MM-HTAI architecture

In this subsection, we describe the imaging system and procedures. Fig. 3.2 shows the so-called MM-HTAI architecture based on the VAST BioImager as designed for zebrafish high-throughput imaging. One at a time, the specimen is loaded into a dedicated capillary. A system of stepper-motors is used to fix and rotate the capillary; the positioning of the specimen in the field of view of the microscope is accomplished with an extra camera (Allied Vision Systems, Pro Silica GE 1050) which is part of the VAST BioImager system. We refer to this camera as the VAST camera which has a mounted lens that observes the capillary via a prism. Originally, the VAST camera is used for visual detection of the location and orientation of the specimen in the capillary so that the system will be able to manipulate the position and perspective of the specimen and initialise the imaging.

In practice, the VAST BioImager can operate with bright-field, fluorescence and confocal microscopy. In a synchronised acquisition protocol, images from different views, e.g, lateral, dorsal, and ventral, are acquired with the microscope mounted

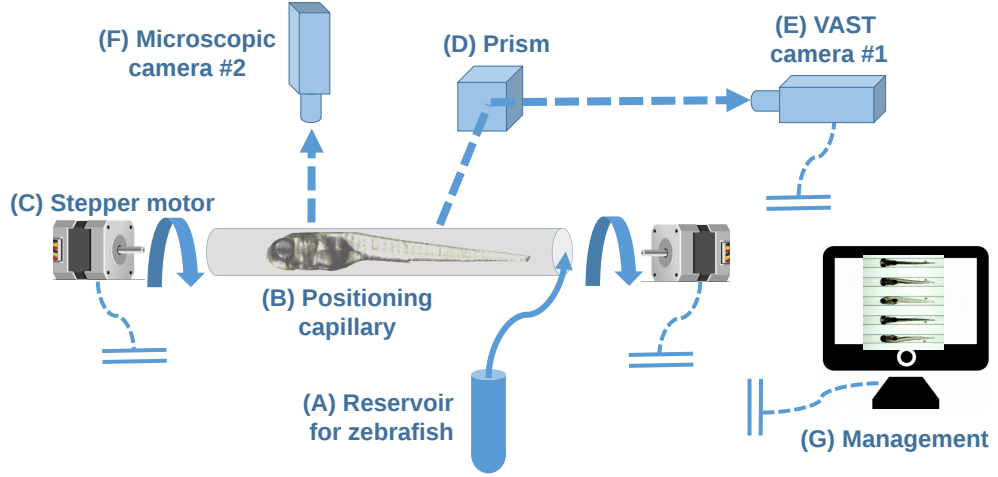


Figure 3.2: A schematic illustration of the MM-HTAI architecture based on the VAST BioImager. Zebrafish larvae are loaded from a reservoir (A) and delivered in to the capillary (B). The stepper motors (C) manipulate the view of the specimen. Through a prism (D), the VAST camera (E) detects the position and orientation of an object and then keeps the object in its field of view. The whole system is mounted on a microscope, of which an equipped high resolution camera (F) facilitates both of the organ- and cellular-level imaging. In addition, the VAST camera (E) manipulated by the management software (G) can acquire arbitrary axial-view images which always present an overview of the zebrafish.

camera or photomultiplier (in case of CLSM) in a full revolution of the capillary. The microscope view renders more detail, due to the characteristics of its lenses, but does not necessarily produce a complete view of the specimen. In addition, the VAST camera can also acquire images of the specimen, at a lower resolution, but always with a full view of the specimen. In fact, it can do so rather efficiently for a large amount of specimen. This is very suitable for our application of 3D reconstruction and measurements of the specimen. Therefore, we adopted the system to be the basis of our 3D reconstructions. It should be noted that the VAST BioImager is mounted on a microscope, of which the high resolution camera will be able to produce more detailed images of the specimen. In combination with the VAST camera, the further analysis of cellular-, organ- and overview-level of the specimen will become possible. This gives rise to the MM-HTAI architecture. In this chapter, we only use the modality of the VAST camera in our MM-HTAI to produce bright-field images.

3.3.2 Preprocessing and segmentation of the images

In our method, we require the binary representations of the object segmented from the colour images acquired from the VAST BioImager. In order to obtain a good and solid reconstruction, these binary representations should reflect the whole subject; in the case of transparent zebrafish, we have to ensure that the translucent parts are also included in the shape representation. We concluded that standard segmentation methods are not sufficient and therefore we have implemented a hybrid method that incorporates the mean shift algorithm (MS) [29] and an improved level-set method (ILS) [87]. In Chapter 2, one can observe that the MS preserves a whole shape representation for the object, but fails in edge sensitivity for the original clear boundaries. The ILS can obtain a more compact 2D shape but is hampered by the problem of edge leakage. So, we combine the two segmentation methods followed by a refinement to obtain accurate segmentations of the object, i.e., the zebrafish. For the rest of the chapter, the results from this segmentation method will be the basis for the 3D reconstructions. The segmentation of a set of axial views will be referred to as the shapes.

3.3.3 Camera model parameterisation

Key to the shape-based 3D reconstruction is a feasible and sufficiently accurate parameterisation of the camera imaging system. For our particular application, the camera position is static while the object rotates through the revolution of the capillary that holds the object, over a given profile axis. In order to make parameter parameterisation and visualisation feasible, the camera is represented as a range of fixed cameras around the object in a circular path as shown in Fig. 3.1(D). Now, for each of these cameras, the pinhole imaging principle can be used to interpret the imaging procedure. We start from a straightforward case of the camera model in which case the camera centre is defined as the world origin. Let $\mathbf{X} = (X, Y, Z, 1)^T$ be a point in 3D space and $\mathbf{x} = (x, y, 1)^T$ be the corresponding point in a 2D image plane, represented in homogeneous coordinates. Then, the mapping between the point and the corresponding image can be geometrically represented as a proportional projection of $x = (fX)/Z$ and $y = (fY)/Z$, where f denotes the focal length of the camera lens. The scaling factors k_x and k_y , image centre $(u_x, u_y)^T$ and the skew factor s are, de facto, the intrinsic properties of the camera. Taking these intrinsic properties into consideration, the matrix representation of the projection can then be formulated as:

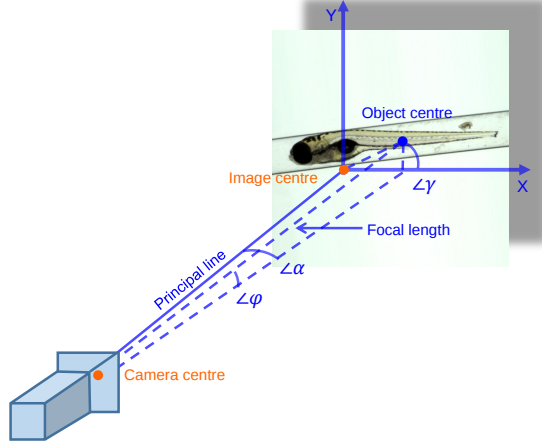


Figure 3.3: A visualisation of the parameterisation of 3D transformation from camera centre to object centre. We assume that the object is positioned in the focal plane and a good quality image is acquired, so the projection line from camera centre to the object centre is defined as the focal length. The $\angle\alpha$ and $\angle\gamma$ represent the 3D rotation angles of camera centre around Y and Z axis. The $\angle\varphi$ is defined as the “translation” angle from the object centre to the image centre. It models the 3D translations of camera centre over Y and Z axis.

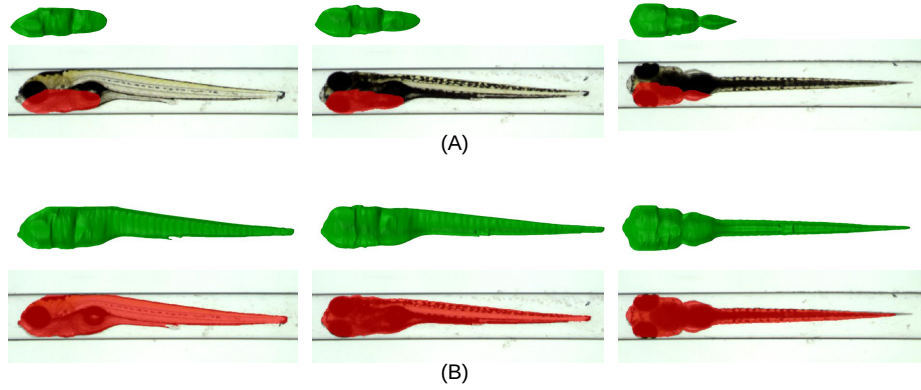


Figure 3.4: Illustration of the importance of camera system calibration. 3D reconstructions of one zebrafish, without (A) and with (B) camera calibration, are depicted. In the first row of (A) and (B) from three different viewpoints are shown. The overlap between the projected image from the 3D models and the original image are shown in red in the second row of (A) and (B). (A) Using an uncalibrated camera configurations results in poor 3D reconstruction and thus a relatively small overlap. (B) Using a calibrated camera system generates an accurate and natural 3D shape. It can be appreciated that projecting the 3D shape to the original axial view results in an almost perfect overlap with respect to the original object.

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$$\tilde{\mathbf{x}} = \begin{pmatrix} fk_x & s & u_x \\ 0 & fk_y & u_y \\ 0 & 0 & 1 \end{pmatrix} \begin{pmatrix} 1 & 0 & 0 & 0 \\ 0 & 1 & 0 & 0 \\ 0 & 0 & 1 & 0 \end{pmatrix} \mathbf{X}, \quad (3.1)$$

where $\tilde{\mathbf{x}} = \mathbf{x}Z$. To simplify the formulation, an implicit transform of Eq. (3.1) is denoted as $\tilde{\mathbf{x}} = \mathbf{P}\mathbf{X}$, where $\mathbf{P} = \mathbf{K} (\mathbf{I} \mid \mathbf{0})$, and $\mathbf{P}$ is defined as the camera projection matrix and $\mathbf{K}$ is defined as the intrinsic camera matrix. In practice, the more generic case for the camera model is to define the object centre as the world origin, which is more convenient for computation and parameterisation. The decomposed parameterisation for the camera model allows convenient incorporation of the extrinsic camera parameters which are the 3D transformation of the camera centre with respect to the world centre.

Suppose that for one object we have N views, the intrinsic camera matrix $\mathbf{K}$ is shared by all the cameras, however, a corresponding 3D transformation for each individual view should be formulated. This is illustrated in Fig. 3.1(C) and Fig. 3.3. For the i^{th} camera, the 3D translation of the camera centre from the object centre is parametrized as $\mathbf{C}_i = [0, f \cos(\varphi_i), f \sin(\varphi_i)]^T$ following a general definition [85]. Now, let $\mathbf{R}_i = \mathbf{R}_{i,X}\mathbf{R}_{i,Y}\mathbf{R}_{i,Z}$ be the total 3D rotation, the revised formulation of the camera projection matrix is then represented by $\mathbf{P}_i = \mathbf{K} \mathbf{R}_i (\mathbf{I} \mid \mathbf{C}_i)$.

The “translation” angle of φ and the rotation angle of γ around Z axis are specified by each of the cameras. In this manner we intend solving the problem that the rotation axis is not exactly parallel to the object centre. In practice, with the VAST BioImager one can accurately align the object, so that these two angles can be shared by all N cameras. Finally, a camera parameter vector is generated through the concatenation of all parameters.

$$\psi = \underbrace{(f, k_x, k_y, u_x, u_y, s)}_{intrinsic} \underbrace{(\alpha, \gamma, \varphi, \omega_{1:N-1})}_{extrinsic}^T. \quad (3.2)$$

Given a specific camera parameter vector ψ , a series of correlated camera configurations can be generated, so that the multi-view 3D projections $\mathbf{P}$ can be modelled.

3.3.4 Shape-based zebrafish 3D reconstruction

We aim to recover the volumetric representation of the object from a range of 2D axial-view images. The shape-based 3D reconstruction method shows to be suitable for shape preservation in this application. Given a series of (object) shapes extracted from the axial-view images, combined with the corresponding camera projection matrices, the 3D representation can be generated by back-projecting the shapes to the 3D world frame so as to obtain the intersections.

Let the 3D point $\mathbf{X}^j \in \mathcal{X}$ ($j \in [1, M]$) be the index of the j^{th} voxel candidate centre included by a collection of voxels, where M is the total number of the voxels; if the collection of voxels is spatially constrained, then M will determine the 3D volumetric resolution. The larger the M is, the higher the resolution will be. Next, one can find the corresponding pixel of the j^{th} voxel in the i^{th} image plane by $\mathbf{x}_i^j = \mathbf{P}_i \mathbf{X}^j$. According to the space-carving theorem, if the voxel $\mathbf{X}^j$ is visible to the view of i^{th} camera, its corresponding image should be covered by the i^{th} shape $\mathbf{S}_i$. In mathematical terms this can be formulated as:

$$\mathcal{X}_i = \{\mathbf{X} \mid \mathbf{S}_i(\mathbf{x}_i^j) \neq 0, \mathbf{x}_i^j = \mathbf{P}_i \mathbf{X}^j, \mathbf{X}^j \in \mathcal{X}\}, \quad (3.3)$$

where $\mathcal{X}_i$ denotes the volumetric representation for the object generated from the i^{th} camera. When involving all the views, we can locate the pixel location in each image plane for each voxel. In general, only the voxels which are visible to all of the N shapes are preserved. Thus,

$$\mathcal{X}^* = \bigcap_{i=1, \dots, N} \mathcal{X}_i \quad (3.4)$$

where $\mathcal{X}^*$ denotes the optimal 3D volumetric representation of the object. Eq. (3.4) can be efficiently implemented by iteratively discarding the invisible voxels from the original voxel collection. However, regarding the eventual imperfection in the shapes segmentation, some voxels which should be included in the 3D object are excluded during this space carving operation. Instead, we assign a score to each of the voxels indicating its visibility. This score is formalised as

$$\mathbf{V}^j = \sum_{i=1}^N \mathbf{1}[\mathbf{S}_i(\mathbf{x}_i^j) \neq 0] \quad (3.5)$$

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where $\mathbf{1}[\cdot]$ denotes an indicator function which takes the value of 1 if the image of the j^{th} voxel lies in the i^{th} shape and 0 otherwise. The score function assigns a value between 0 and N for each individual voxel. If, in addition, a constrained error tolerance rate ϵ is defined, the problem of imperfection in the shape segmentation can be more or less solved by thresholding the membership function of a voxel. Accordingly, the 3D reconstruction model of Eq. (3.4) is reparameterised as:

$$\mathcal{X}^* = \{\mathbf{X} \mid \mathbf{V}^j \geq (1 - \epsilon)N, \mathbf{X}^j \in \mathcal{X}\}, \epsilon \in [0, 1). \quad (3.6)$$

For our particular application in zebrafish imaging, we have empirically established that the parameter ϵ takes the value of 0.05, meaning that the voxels visible up to 95% of the shapes will contribute to the 3D reconstruction. With the reconstructed 3D volumetric representation, an initial 3D surface model can be obtained by the marching cubes algorithm; this surface model is formulated as dense surface points and a triangulated mesh, which can be further optimised [86].

3.3.5 Camera system optimisation

From the previous sections it becomes clear that the camera projection matrix, i.e., the camera configuration, plays an important role in the shape-based 3D reconstruction. A good estimation of the camera configuration will ensure accuracy in the 3D projective geometry. If a set of specific camera parameters as denoted in Eq. (3.2) are given, the camera projection matrix $\mathbf{P}$ can be correspondingly computed. The camera parameters, more specifically, the extrinsic camera properties, are usually unknown or not sufficiently accurate; e.g., through a drift of the image centre from the object centre. Therefore, an automated camera configuration estimation, i.e., a camera calibration, should be performed. The importance of a camera calibration is demonstrated in Fig. 3.4 by the results of applying the method to 3D reconstruction of zebrafish shapes. Standard camera calibration methods [88, 89] for our imaging environment are not available for the small scales of microscope imaging, i.e., the VAST camera. Therefore we have proposed [40] a method similar to ideas on texture registration and stitching [84, 85]. We define the area coherence as the area of overlap between the image projected from the

3D reconstructed model and the groundtruth shape. Accordingly, we can define an energy function as:

$$f(\psi) = \frac{1}{N} \sum_{i=1}^N C(\mathbf{S}_i, \mathbf{P}_i(\psi) \mathcal{X}), \quad (3.7)$$

where the camera matrix is reparameterised as $\mathbf{P}_i(\psi)$ meaning that $\mathbf{P}$ is a function of ψ . By evaluating the camera configuration space, the optimal suggestion of ψ can be obtained by maximising Eq. (3.7)

$$\psi^* = \arg \max_{\psi \in \Psi} f(\psi). \quad (3.8)$$

Visualisations corresponding with this approach are depicted in the lower rows of Fig. 3.4 (A) and (B). We argue, however, that the expression of Eq. (3.7) is equivalent to the, so called, voxel residual volume (VRV) which is defined as

$$f(\psi) = |\mathcal{X}^*(\psi)|. \quad (3.9)$$

Eq. (3.9) denotes the total number of the voxel candidates of the 3D volumetric representation. Similarly, the reparameterisation of $\mathcal{X}^*(\psi)$ indicates the functional relation between $\mathcal{X}^*$ and ψ . In fact, keeping the maximal total overlap area between the projected images and the groundtruth shapes is equivalent with keeping the maximal VRV of the reconstructed object. The VRV is, however, computationally more efficient while the same performance can be obtained. This is because the computation of the area coherence is unnecessary. For such an unconstrained optimisation problem, multiple optimisers are available, for example, the Nelder-Mead simplex [90] and the Evolution Strategy (ES) [91, 92]. One should be aware that, although small, there always exists the risk of a local minimum of the optimisers which may lead to an inaccurate camera configuration estimation. However, feasible initialisations are possible so as to provide accelerations and improvements to the optimisation. The intrinsic camera parameters including the focal length and the scaling factors are provided by the camera and lens specifications. The extrinsic parameters including the 3D rotations and translations can be assigned through reasonable estimations; e.g., the parameter of ω_i is initialised as $\omega_i = (i - 1) * 2\pi / N$. With these sensible initialisations, more efficient and accurate camera configurations become possible.

3.4 Experimental results

In this section we explore the method through validation experiments. In Subsection 3.4.1, we define the sampling for both the axial-view and spatial elements. In Subsection 3.4.2, we perform the experiments on calibration particles to determine the sampling size and validate the accuracy of proposed method. In Subsection 3.3.3, we evaluate the performance of our method on zebrafish larvae. This will result in numerical and statistical validity of the method so that it can be successfully applied in further research. In Subsection 3.3.4, we report the 3D measurements of volume and surface area using a statistical representation for the zebrafish in three larval stages.

3.4.1 Sampling of axial-views and volume for the experiments

With respect to the sampling, the physical limitations imposed by the hardware and the imaging scale are confined. There are two sampling modes that cooperate in the reconstruction process. The production of axial-view images is depending on the properties of the hardware, which is referred to as the Axial-view Sampling Density (ASD) and determined by the step size of the stepper motors that operate the capillary. For the experiments we will use a range of steps in order to find a good operational ASD from sparse to dense. For the shape-based 3D reconstruction, a volumetric representation is required. From the hardware and the spatial constraints of specimens, a confined volume of $5000 \times 1000 \times 1000 \mu m^3$ (width $\times$ height $\times$ depth) is determined, which can include the whole object. The sampling involves the variation of the size of the isotropic elements (voxels) in this volume, which can be inferred as the total number of the elements. The smallest sampling size will be equal to the sampling elements on the CCD sensor in which case the number of sampling elements is 30×10^6 ; this represents the largest number of sampling elements. The smallest number of elements is 1×10^6 which gives rise to a considerably different performance. The given sampling volume is used in all experiments described in this chapter.

3.4.2 Validation of the proposed method

In order to validate the accuracy of the proposed method, particles, i.e., beads, with a known size distribution are used (GP 500 μm Control Particles, High

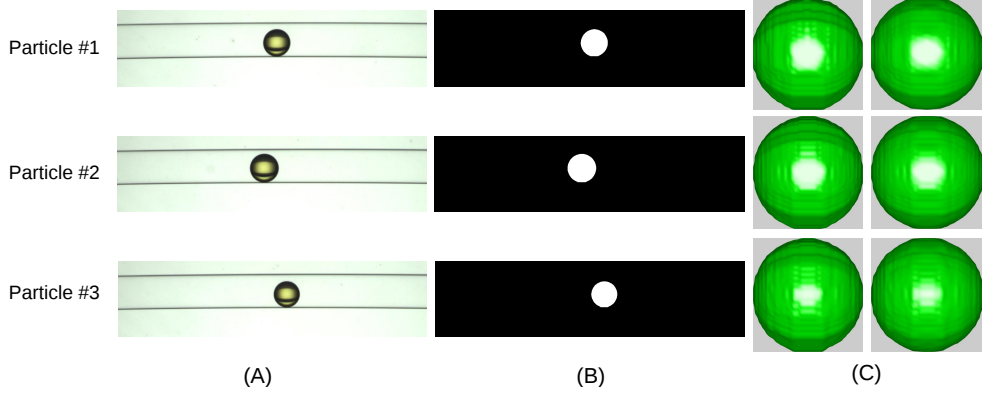


Figure 3.5: Illustration of example images of 3 calibration particles and their 3D reconstruction. Each row indicates one calibration particle example. (A) Original RGB images. (B) Extracted profiles. (C) Reconstructed 3D models by an ASD=7 (left column) and an ASD=21 (right column). Although the ASD=7 already produces acceptable 3D shapes for the calibration particles, while some carving artifacts are still visible. A better result is shown for the 3D models reconstructed with an ASD=21.

Fluorescence, lot 110701, Union Biometrica). The particles are used to calibrate the VAST BioImager system. Some typical examples are depicted in Fig. 3.4(A), from which we can observe some variation in the diameter of the particles. We assume that this variation in size is according to a Gaussian distribution as given by the manufacturer, i.e., $\mathcal{N}(500, 25)$ measured in μm . We have acquired image sets from the VAST BioImager for 25 calibration particles. Subsequently, the proposed 3D reconstruction method is applied to these image sets.

If the size measured from the reconstructions corresponds with the distribution of the size of the original calibration particles, it means that the proposed method can accurately recover the 3D object in terms of size and shape. There are two methods for evaluations: (A) To measure the diameter of the reconstructed calibration particles, a 3D sphere is estimated to fit the dense surface points by minimizing a least squares function. (B) To measure volume and surface area, the reconstructed 3D models can be used.

The result of the shape-based 3D reconstruction method can be affected by the Axial-view Sampling Density (ASD) and the voxel size (sampling density). As far as the ASD is concerned, more views may result in a more accurate and natural shape, similarly a higher sampling density of the voxels may result in a more accurate representation of the shape. Therefore, the experiments are designed to

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verify the accuracy according to these two parameters as well as establishing the correctness of the measurement itself by comparing to analytical representations or empirical observations.

The results are presented in Table 3.1 to 3.3; here the sampling density is denoted as SD given the size of the isotropic sampling element (in μm). For all tables the horizontal direction, from left to right, indicates an increasing sampling density from $17.10 \mu m$ per voxel to $5.85 \mu m$ per voxel. The vertical direction indicates an increase of the ASD from 4 to 84 views. We have configured our imaging system to take 84 images, corresponding with a step-size of 4.3° per axial-view, in a full revolution of the shape. Initial experiments have shown that a strategy of even axial-view sampling in a full revolution accomplishes the most stable performance. By resampling of the 84 views of 4.3° , a range of different ASDs is obtained for the experiments.

(A) Evaluations for diameter of the calibration particles

We interpret the measurements of the diameter in terms of the mean and standard deviation over the 25 calibration particles and present the results in Table 3.1. The results show that from the proposed method accurate estimations are obtained which are in range of the distribution ($500 \pm 25 \mu m$) provided by the manufacturer. The results vary with different ASD and SD. More specifically, an increase in the sampling density results in an increase of the estimated diameter which asymptotically stabilises at the higher sampling densities. A high sampling density generates a smoother 3D surface, while for lower resolutions the volumetric representation tends to be a little bit expanded. An increase of the ASD results in a decrease of the estimated diameter while for higher ASD it stabilises. Using more views to reconstruct the 3D shapes causes less voxels to be preserved and the 3D shapes seem to be more compact compared to the ones reconstructed by smaller ASD. We tested whether our measurements were sufficiently accurate. In Table 3.1, in each second row, the T-test scores are given per ASD. We state in the null hypothesis that the measured diameter from the 3D reconstructed models equals to the values (mean, sigma) given by the manufacturer, i.e., $500 \mu m$. For a significance level of 0.01 (two-sided T-test), a value of 2.787 is given from the table of selected values. From Table 3.1 one can appreciate that most T-test scores are smaller than the selected value; this means that our measurements for the diameter of the calibration particles from the 3D

reconstructions are accurate. Especially, the values from the ASD of 21 combined an SD of $6.93 \mu m$ or smaller come out very well.

(B) Evaluations for volume and surface area of the calibration particles

The calibration particles have been provided with an indication of the size distribution, i.e., given diameter. However, the real distributions of the volume and surface area are unknown. In Table 3.2 and 3.3 the volume and surface area of the calibration particles are presented as computed from the reconstructed shapes in a range of parameters for ASD and SD. Volumes are computed directly from the voxels in the shape, i.e., integrating over the residual voxels after reconstruction. For each object a surface model is composed from a dense surface point cloud and a mesh triangulation from these points. The surface area can be obtained by integrating over the facets of the meshes using Heron's formula [86] $A = (s(s-a)(s-b)(s-c))^{(1/2)}$, where $s = (a+b+c)/2$ and a, b, c represent the edge-length of a triangle. We assume that the shape of the calibration particles is a standard sphere, consequently the diameters from Table 3.1 are used to obtain the analytical computation of the volume and surface area by $V = (4/3) * \pi (d/2)^3$ and $S = 4\pi (d/2)^2$ for each set of parameters.

For each ASD, the values measured from the reconstructions are listed in each first row of Table 3.2 and 3.3, indicated by M. The second row for each ASD in Table 3.2 and 3.3 lists the analytical computations, indicated by A. The trends in Table 3.2 (Volume) and 3.3 (Surface area) are similar to those observed in Table 3.1. While the ASD increases, the reconstructed shapes are smoother and less voxels are preserved. Moreover, the variation in the results decreases at higher SD with voxels smaller than $6.93 \mu m$.

Taking the reconstruction effects shown in Fig. 3.5 (C) into account, it can be appreciated that a ASD of 21 is a reasonable choice to generate a smooth and natural shape with very few carving artifacts. Moreover, one can observe rather small differences between the measured and analytical values for an ASD of 21 as of the SD of $6.93 \mu m$; i.e., the shape of the calibration particle is well preserved and reconstructed with such set of parameters. In comparison, a larger value of ASD requires more camera projections and the 3D reconstruction is therefore computationally more expensive; a sampling density smaller than $6.93 \mu m$ requires more memory and computation to ensure the precision. So, the results in this experiment indicate that the proposed method can obtain accurate 3D

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reconstructions measured in size, volume and surface area; the ASD of 21 and SD of $6.93 \mu m$ are empirically selected as the configurations for the further experiments.

3.4.3 Evaluations on zebrafish larvae

In order to obtain 3D reconstructions and evaluate the performance of the proposed method as applied to zebrafish specimens, we first assemble a large number of image sets of zebrafish larvae with the help of the axial-view imaging architecture presented in Subsection 3.3.1 and put the images in a dataset. We have chosen the most frequently used larval stages (for VAST BioImager) to be represented in the dataset; meaning 3 groups, i.e., 3, 4 and 5 days post fertilisation (dpf), containing 12, 24 and 24 instances, respectively. Although the VAST BioImager is suitable for live imaging, for this experiment, we have used fixed samples of one and the same strain, i.e., ABxTL wild type. All samples were fixed in 4% PFA/PBS (PFA: paraformaldehyde; PBS: Phosphate-Buffered saline). For the imaging, the samples were kept in a solution of PBS with 0.3% Tween-80.

For each specimen, the same imaging setup of the VAST BioImager is consistently used (cf. calibration experiment configuration). Specifically, in one full revolution, 84 views are captured with equal step size. Other ASDs, i.e., 42, 21, etc., are evenly sampled from the these 84 views. In the acquisition, the rotation steps between any two contiguous views are considered to be equal. However, due to the drift on the electro-mechanical parts of the VAST imager, we have to take into account that there can be a variation in the step size. If present, this variation can seriously affect the 3D reconstruction of the zebrafish specimens and therefore a calibration of the camera system is necessary.

In Table 3.4 and 3.5, we list the volume and surface area for the three groups of larval stages. For these tables the horizontal direction indicates an increase in ASD. In order to get a better idea of the data, we visualised Table 3.4 and 3.5 in Fig. 3.6 and 3.7, in which the voxel residual volume (VRV) and the surface area are plotted as a function of the ASD, respectively.

For all three groups the trend is similar, as the ASD increases the VRV decreases. This can be explained from the methodology as only voxels which are visible to most of the views are preserved. Consequently, less voxels will be preserved when more views are employed. The decrease of VRV rapidly changes in an

asymptotic fashion of VRV for an ASD larger than 7. The asymptote indicates that we do not need an abundance of sampling views, an ASD of 42 or 84 does not result in a much better reconstruction and therefore these are unnecessary for the reconstruction of the zebrafish larvae in our application.

The VRV changes with the growth of the zebrafish for the different larval stages; i.e., as the zebrafish grow, the VRV increases. Similar trends can also be found in Fig. 3.6 which depicts the asymptotics of surface area for the 3D zebrafish models. External conditions influence growth as it also depends on the feeding policy. At some point in development the larvae have used all their yolk and are thereafter depending on nutrients in the medium. So, if the amount of nutrients is low, a delayed growth will occur.

From Fig. 3.6 and 3.7 it can be concluded that an ASD of 7 is already a good option for this application. However, integrating the results from all experiments, shows that an ASD of 21 achieves the best performance measured in terms of size, volume and area. In order to appreciate this, the reconstructed results are visualised for an identical zebrafish specimen in Fig. 3.8. For a ASD of 4 or 7, the 3D models exhibit a large number of artificialities. The space carving effects generate some flat regions on the surface. However, with a higher ASD value, such as 12 or 14, the 3D reconstruction results improve; the surface of the shape is still rough though. As of an ASD of 21 or larger, one can appreciate that results are more or less similar; the 3D shapes all appear smooth and natural. The visualisation quality does not improve much for an ASD larger than 21, while a large ASD is computationally much more expensive. The empirical ASD of 21 is therefore a motivated tradeoff over a range of considerations regarding the 3D reconstruction of zebrafish larvae and the measurement thereof.

A number of typical examples of models from 3D reconstructed zebrafish larvae are shown in Fig. 3.9. For each zebrafish developmental stage in our experiment two individuals have been selected; the 3D shapes are visualised with and without texture mapping. The texture mappings are just serving visualisation purposes, where texture is used to add some realism to the view. The specimens, in this stage, are still partially transparent, therefore, some artificial mapping was applied to obtain a realistic visualisation. For measurement the binary volume models are used.

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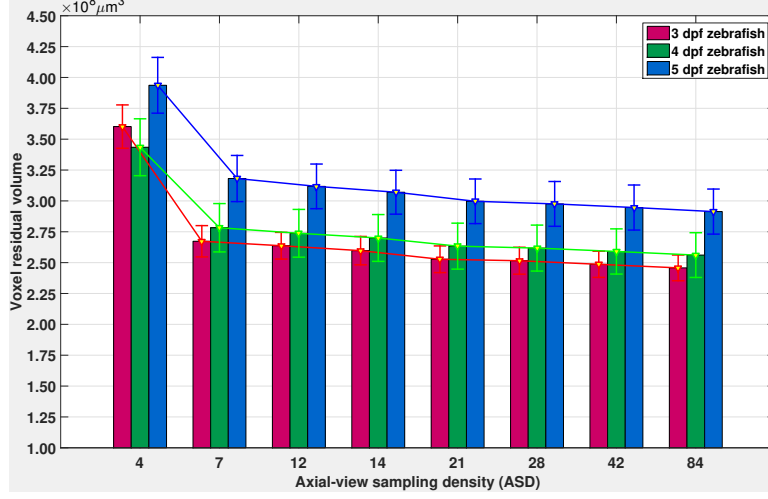


Figure 3.6: Voxel residual volume of zebrafish larval stages against ASD. The descending trends of the voxel residual volume are clearly demonstrated in the graph for all 3 groups of zebrafish larvae at increasing ASD. However, after an ASD of 7 the decrease tends to be asymptotic, especially after an ASD of 21. This means that dense axial-view sampling (42, 84, etc.) for our 3D zebrafish reconstruction and measurements is unnecessary. The consistent differences of the volume for the three larval stages can be observed.

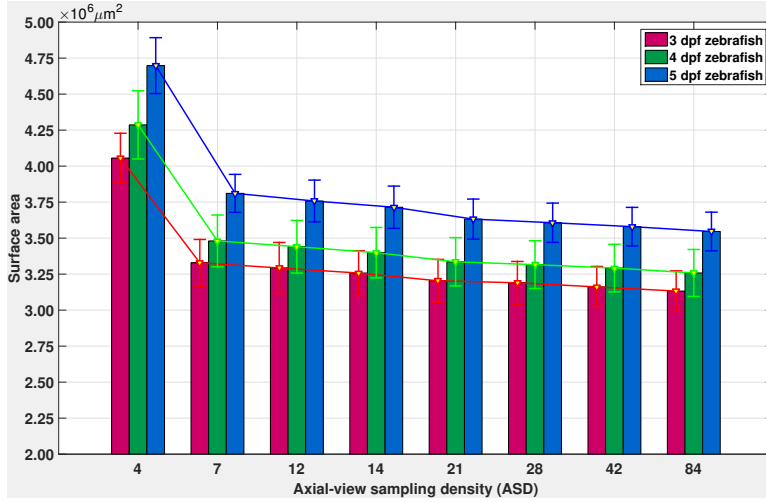


Figure 3.7: Surface area of zebrafish larval stages against ASD. The trend in surface area is similar to that of the volume as depicted in Fig. 3.6. The surface area is computed from a mesh, to suppress noise a mesh smoothing, i.e., implicit fairing, with 10 iterations was applied to all meshes [93].

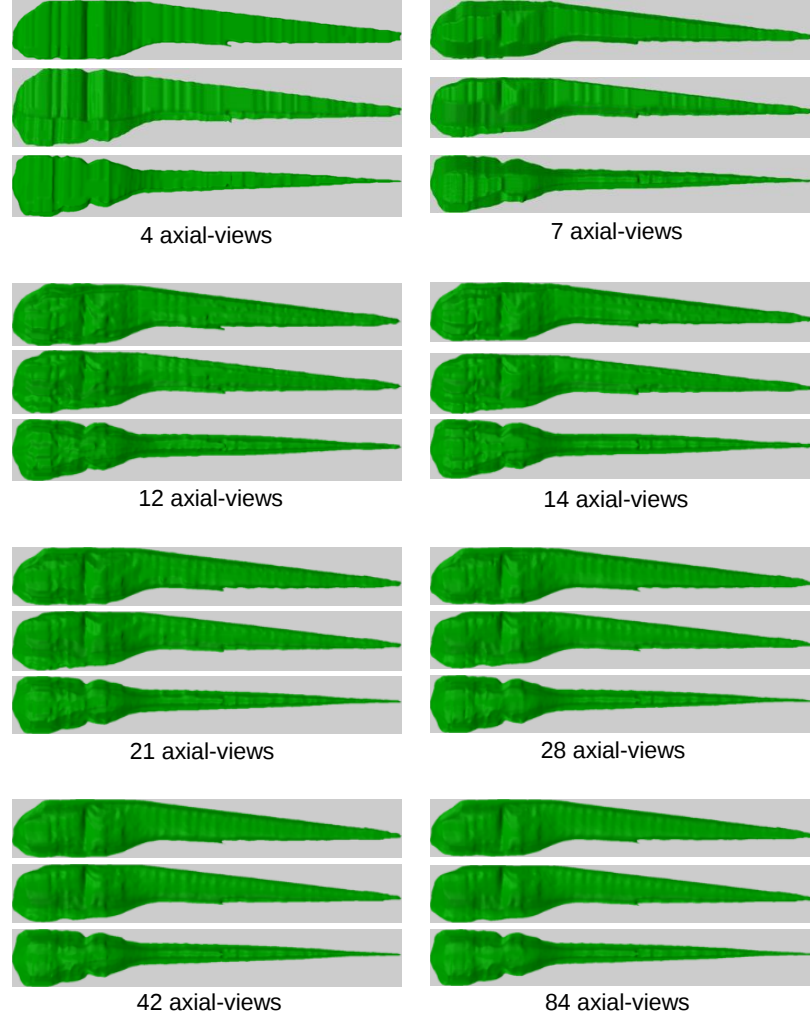


Figure 3.8: Visualisations of 3D reconstructions for a range of different ASD using the same zebrafish larva instance. The use of an ASD=4 (A) and an ASD=7 (B) results in the 3D reconstructions presenting a large number of sharp and flat surface elements which are generated by the carving effects of shape-based 3D reconstruction method. The use of an ASD=12 (C) and an ASD=14 (D) results in an improvement of the 3D models, but some carving artifacts remain. Using an ASD=21(E) to an ASD=84 (H) results in accurate and natural-shaped 3D models. For our particular problem domain, this suggests that both sparse and dense axial-view sampling are not the optimal configuration for zebrafish larvae 3D reconstruction. Sparse axial-view sampling produces poor 3D models while dense axial-views sampling requires more computation time whilst the effects do not improve accordingly.

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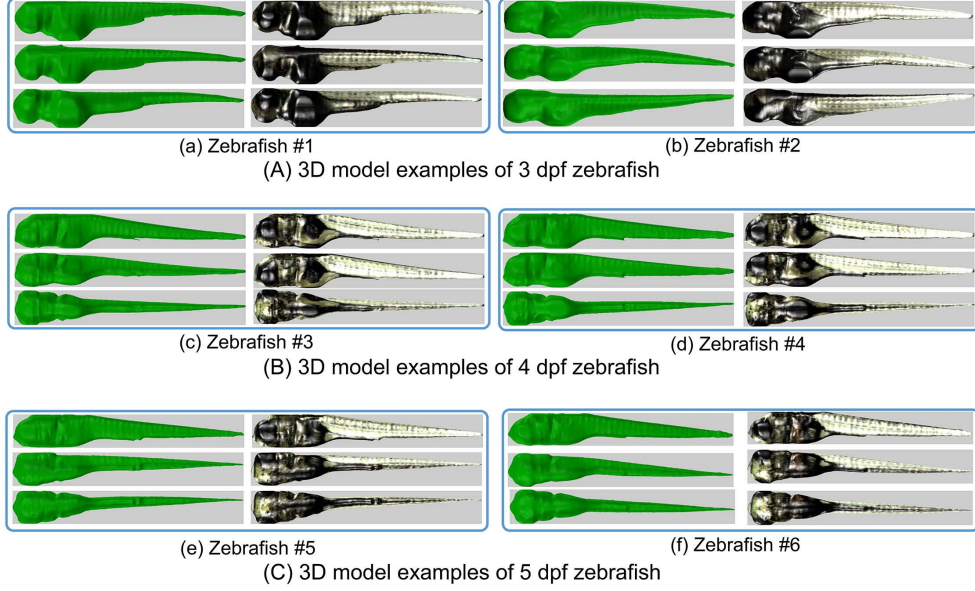


Figure 3.9: Visualisation of 3D models of 3 zebrafish larval stages (3 dpf, 4 dpf and 5 dpf). Each box represents a reconstructed 3D model for one specific zebrafish larvae visualised from three different viewpoints. The 3D volumetric representations are shown on the left side in each box. The models with texture-mapping are in the right side of each box. (A) 3D models of two selected 3 dpf zebrafish larvae. (B) 3D models of two selected 4 dpf zebrafish larvae. (C) 3D models of two selected 5 dpf zebrafish larvae. Variation in size and shape between stages and within stages (interclass and intraclass) can be appreciated from the visualisations. A remarkable intraclass discrimination originates from the size and colour of the yolk. In addition, animations of the 3D zebrafish models are available at: <http://bio-imaging.liacs.nl/galleries/VAST-3Dimg/>

3.4.4 3D measurements on zebrafish larvae

From the aforementioned experiments we may conclude that (1) an accurate reconstruction can be obtained from our method and that (2) from the 3D reconstruction accurate measurements can be made. In order to assess the shape and size in a high-throughput setup we use the database of our samples to find the distributions per larval stage.

From our empirically established parameters we reconstruct our images to 3D models using an ASD of 21 and a voxel sampling density of $6.93 \mu m$. On these models, the computations are performed, the volume and surface area expressed in μm^3 and μm^2 respectively. Over the measurements per group, the mean and

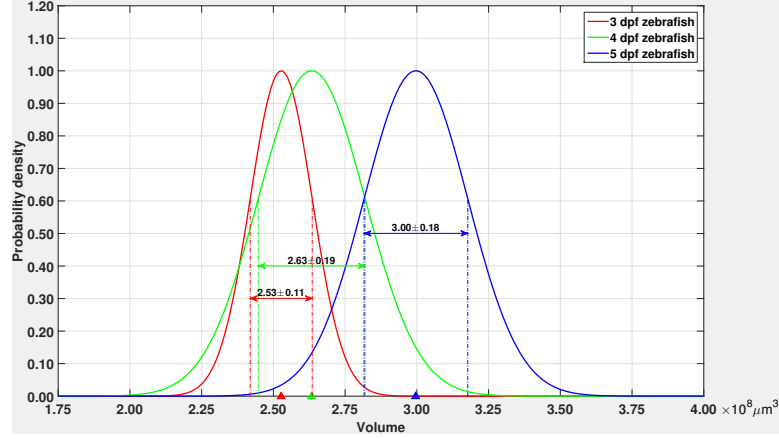


Figure 3.10: Distribution of volume of zebrafish larval stages (3 - 5 dpf). X axis: volume; Y axis: normalised probability density. The colour-filled triangles on the Volume-axis indicate the locations of the mean for each of the 3 distributions. The numerical values of the mean and standard deviation are indicated with corresponding double-sided coloured arrows. One can see that the volume of older zebrafish larvae is always larger than the younger ones. The growth rate increases more from 4 dpf to 5 dpf compared to that from 3 dpf to 4 dpf.

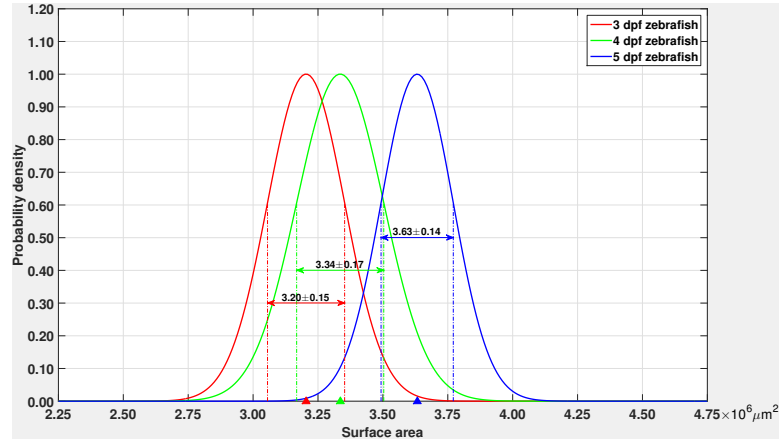


Figure 3.11: Distribution of surface area of zebrafish larval stages (3 - 5 dpf). X axis: surface area; Y axis: normalised probability density.

standard derivation are calculated and a Gaussian distribution is applied to model the data. In order to compare the 3 groups of larval stages the density distributions are normalised.

The distribution of the volume is shown in Fig. 3.10. The trend as seen in Fig 3.6 is reflected in the results in that the volume of 5 dpf zebrafish is the largest, and

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that of 3 dpf zebrafish is the smallest. The volume is more discriminative for the 3 dpf and 5 dpf zebrafish. The 3 dpf and 4 dpf zebrafish appear to be very similar in volume, which can be seen from the large overlap of the two distributions. Indeed, these larval stages are quite similar in shape and appearance and this is also illustrated in Fig. 3.9. The distribution of surface area is shown in Fig. 3.11 and can be interpreted in similar fashion to the volume measurement. A plot for the joint distribution of volume and surface area is shown in Fig. 3.12. Although the distributions of 4 and 5 dpf zebrafish are close to each other we can clearly separate the 3 centres.

This experiment provides significant quantitative information on zebrafish larvae because from the distributions, given the larval stage, the regularity of the shape can be directly assessed. The distributions provide an absolute reference baseline of volume and surface area for zebrafish in 3 larval stages. This is very important for applications in toxicology and drug targeting in which quantities of specific substances need to be related to volume and shape and phenotypical information on dose and effect. These need to be derived from measurements on the specimen. Moreover, as the readout of effects of substances is often measured with fluorescent probes, a relation to volume is even more important. In addition to these basic measurements other 3D features, i.e., moments, wavelets, shape descriptors, etc., can be considered. This will further strengthen the phenotypical description and assessment of zebrafish larvae in high-throughput imaging applications.

In order to make sure that we could measure the specific characteristics of the given larval stages, we used fixed specimens. In a control experiment we have used living larvae staged by specialists and imaged with the VAST BioImager under exactly the same conditions. We used 7 examples from 4 dpf and 8 examples from 5 dpf. The results of the measurements are shown in Table 3.6. From these results one can appreciate that they are all within the range of the fixed specimens.

We validated our methodology for the 3D reconstruction and measurements and at the same time showed the importance of the baseline measurements for the 3 larval stages; the major stages in compound screens with zebrafish. The reference values for volume and surface area as well as their distributions for the different larval stages can be directly used in experiments. Consequently, for phenotype analysis an assessment for relative size and shape can be made. Moreover,

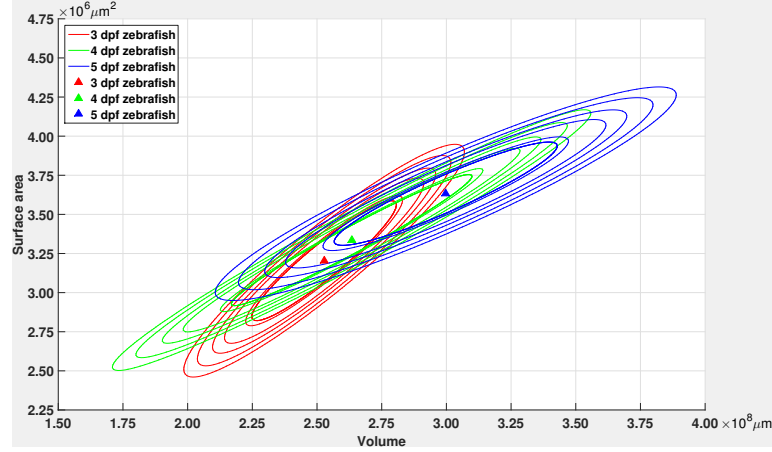


Figure 3.12: Joint distribution of volume and surface area of zebrafish larval stages (3 - 5 dpf). X axis: volume; Y axis: surface area. The 3 joint distributions have overlap with respect to each other, especially for the 4 dpf zebrafish larvae. Nevertheless, individual distribution centres can still be separated. The colour schema is similar to that of Fig. 3.10 and Fig. 3.11

features that are frequently used for phenotyping zebrafish and thereby evaluating anomalies can be related to size and shape to better assess the effect under study.

3.5 Chapter conclusions and future work

In this chapter, we presented the multi-modal high-throughput axial-view imaging (MM-HTAI) architecture based on the VAST BioImager in which images of zebrafish larvae are acquired. It answers the RQ 1: *To what extent is it possible to develop an MM-HTAI architecture for the zebrafish larvae?* We presented the shape-based 3D reconstruction using the acquired axial-view images to reconstruct 3D models so that 3D shape description of the zebrafish are obtained. Subsequently, 3D measurements can be applied on the whole specimen. It answers the RQ 3: *To what extent is it possible to obtain precise 3D shape description and derive accurate 3D measurements that are statistically relevant for the zebrafish from the MM-HTAI architecture?*

More concrete, the research presented in this chapter integrates the MM-HTAI architecture, image segmentation, camera system optimisation and the shape-based 3D reconstruction method. The 3D measurements of volume and surface area,

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as well as the 3D visualisation become available through the proposed method on zebrafish larvae using high-throughput imaging. The important conclusions are that (1) accurate 3D measurements for the zebrafish larvae can be made in high-throughput; (2) as of now a metrical reference of shape descriptors for 3 important larval stages is produced and (3) the methodology for these measurements is validated.

Below, we propose four directions for future research. (1) The results are directly applicable for a range of different *in vivo* applications, such as toxicology, drug targeting and infection studies. Further research will be directed to 3D shape alignment so that an even better match between different zebrafish instances can be given. Subsequently, other 3D features will be explored to accomplish elaborate classification of subtle differences between different treatment groups of zebrafish in experiments. (2) The VAST camera provides good overview images which have been employed for this study. The same methodology can be applied with the microscope camera. The higher numerical aperture and the magnification of the lenses in the microscope will then provide 3D details of the specimen in similar fashion. The tradeoff of using a confocal or wide field approach for such applications needs to be assessed from future results. (3) Alternative to the axial-reconstruction with the VAST BioImager, Optical Projection Tomography (OPT) imaging can be probed. This imaging approach has been successfully applied in zebrafish research [21] and, recently, successful high-throughput applications in zebrafish have been reported [22]. (4) As indicated, the measurements and their distributions provide reference values for volume and surface area (size and shape). This supports the assessment of other observations as we now can relate the effect of a change in size to a norm. In a similar manner, the VAST system can be used to provide distributions of other morphometrical landmarks in zebrafish development; to this end the microscope can be used to acquire axial-views at higher resolution and, in that manner, provide 3D features. These features can be combined with volume/surface area features to enable relative comparisons between experiments. Such analysis will further enhance our understanding of phenotypes in zebrafish and their exposure to experimental conditions which is a necessity for high-throughput analysis.

Table 3.1: Diameter statistics of calibration particles (M) measured from 3D reconstructed models (μm) and the corresponding (T) T-test scores.

$\frac{SD}{ASD}$	17.10	10.00	7.94	6.93	6.30	5.85	5.50
M	507.20 $\pm$ 26.21	511.07 $\pm$ 25.94	511.74 $\pm$ 25.83	512.08 $\pm$ 25.88	512.33 $\pm$ 25.85	512.42 $\pm$ 25.89	512.42 $\pm$ 25.86
T	1.34	2.09	2.23	2.29	2.34	2.35	2.35
4	494.60 $\pm$ 25.69	499.00 $\pm$ 25.39	499.85 $\pm$ 25.33	500.26 $\pm$ 25.34	500.50 $\pm$ 25.31	500.64 $\pm$ 25.32	500.68 $\pm$ 25.30
7	1.03	0.19	0.03	0.05	0.10	0.12	0.13
12	502.83 $\pm$ 25.85	505.71 $\pm$ 25.55	506.14 $\pm$ 25.50	506.35 $\pm$ 25.54	506.49 $\pm$ 25.49	506.56 $\pm$ 25.50	506.57 $\pm$ 25.57
14	0.31	0.94	1.02	1.06	1.09	1.10	1.10
14	499.70 $\pm$ 25.80	502.98 $\pm$ 25.49	503.48 $\pm$ 25.42	503.75 $\pm$ 25.44	503.90 $\pm$ 25.42	504.00 $\pm$ 25.42	504.02 $\pm$ 25.46
21	0.06	0.57	0.67	0.72	0.75	0.77	0.77
21	493.57 $\pm$ 25.64	497.96 $\pm$ 25.36	498.70 $\pm$ 25.28	499.03$\pm$25.29	499.22 $\pm$ 25.27	499.32 $\pm$ 25.27	499.32 $\pm$ 25.34
28	1.23	0.39	0.25	0.19	0.15	0.13	0.13
28	491.39 $\pm$ 25.63	496.52 $\pm$ 25.35	497.52 $\pm$ 25.27	498.00 $\pm$ 25.29	498.29 $\pm$ 25.25	498.46 $\pm$ 25.28	499.00 $\pm$ 25.37
42	1.65	0.67	0.48	0.39	0.33	0.30	0.29
42	491.93 $\pm$ 25.61	496.88 $\pm$ 25.34	497.84 $\pm$ 25.26	498.27 $\pm$ 25.27	498.52 $\pm$ 25.26	498.68 $\pm$ 25.25	498.73 $\pm$ 25.34
84	1.54	0.60	0.42	0.34	0.29	0.26	0.25
84	490.82 $\pm$ 25.61	496.05 $\pm$ 25.35	497.09 $\pm$ 25.27	497.56 $\pm$ 25.27	497.84 $\pm$ 25.25	498.00 $\pm$ 25.26	498.05 $\pm$ 25.36
	1.76	0.76	0.56	0.47	0.42	0.39	0.38

SD = sampling density; ASD = axial-view sampling density.

Table 3.2: Volume statistics of (M) measurements from the 3D reconstructed models and (A) analytic calculation from the fitted diameter for calibration particles ($\times 10^7 \mu m^3$).

	SD ASD								
		17.10	10.00	7.94	6.93	6.30	5.85	5.50	
4	M	7.073 $\pm$ 1.085	7.076 $\pm$ 1.080	7.070 $\pm$ 1.075	7.072 $\pm$ 1.079	7.074 $\pm$ 1.078	7.074 $\pm$ 1.080	7.070 $\pm$ 1.079	
	A	6.884 $\pm$ 1.077	7.042 $\pm$ 1.083	7.069 $\pm$ 1.081	7.083 $\pm$ 1.083	7.093 $\pm$ 1.084	7.097 $\pm$ 1.086	7.097 $\pm$ 1.084	
7		6.651 $\pm$ 1.021	6.648 $\pm$ 1.015	6.644 $\pm$ 1.014	6.646 $\pm$ 1.015	6.647 $\pm$ 1.014	6.647 $\pm$ 1.015	6.644 $\pm$ 1.018	
		6.385 $\pm$ 1.005	6.554 $\pm$ 1.010	6.588 $\pm$ 1.011	6.604 $\pm$ 1.013	6.613 $\pm$ 1.013	6.619 $\pm$ 1.014	6.621 $\pm$ 1.017	
12		6.530 $\pm$ 1.002	6.534 $\pm$ 0.999	6.530 $\pm$ 0.997	6.530 $\pm$ 0.999	6.532 $\pm$ 0.997	6.531 $\pm$ 1.000	6.528 $\pm$ 1.004	
		6.708 $\pm$ 1.029	6.822 $\pm$ 1.030	6.839 $\pm$ 1.029	6.848 $\pm$ 1.031	6.853 $\pm$ 1.031	6.856 $\pm$ 1.031	6.857 $\pm$ 1.033	
14		6.531 $\pm$ 1.002	6.527 $\pm$ 0.999	6.522 $\pm$ 0.996	6.523 $\pm$ 0.997	6.524 $\pm$ 0.997	6.524 $\pm$ 0.998	6.519 $\pm$ 1.004	
		6.584 $\pm$ 1.029	6.712 $\pm$ 1.030	6.732 $\pm$ 1.029	6.743 $\pm$ 1.031	6.748 $\pm$ 1.031	6.752 $\pm$ 1.031	6.754 $\pm$ 1.033	
21		6.518 $\pm$ 1.000	6.517 $\pm$ 0.998	6.514 $\pm$ 0.995	6.514$\pm$0.997	6.515 $\pm$ 0.997	6.515 $\pm$ 0.998	6.512 $\pm$ 1.002	
		6.345 $\pm$ 0.998	6.514 $\pm$ 1.005	6.542 $\pm$ 1.004	6.555$\pm$1.006	6.563 $\pm$ 1.006	6.567 $\pm$ 1.007	6.567 $\pm$ 1.009	
28		6.449 $\pm$ 0.993	6.449 $\pm$ 0.992	6.443 $\pm$ 0.988	6.443 $\pm$ 0.989	6.446 $\pm$ 0.990	6.444 $\pm$ 0.991	6.439 $\pm$ 0.998	
		6.282 $\pm$ 0.991	6.472 $\pm$ 1.000	6.509 $\pm$ 1.000	6.525 $\pm$ 1.002	6.535 $\pm$ 1.003	6.542 $\pm$ 1.003	6.544 $\pm$ 1.007	
42		6.441 $\pm$ 0.991	6.442 $\pm$ 0.990	6.436 $\pm$ 0.985	6.436 $\pm$ 0.986	6.438 $\pm$ 0.986	6.437 $\pm$ 0.989	6.432 $\pm$ 0.994	
		6.262 $\pm$ 0.989	6.458 $\pm$ 0.999	6.496 $\pm$ 1.000	6.515 $\pm$ 1.002	6.526 $\pm$ 1.002	6.533 $\pm$ 1.003	6.535 $\pm$ 1.007	
84		6.416 $\pm$ 0.988	6.419 $\pm$ 0.988	6.413 $\pm$ 0.983	6.411 $\pm$ 0.984	6.413 $\pm$ 0.984	6.413 $\pm$ 0.988	6.407 $\pm$ 0.995	
		6.240 $\pm$ 0.986	6.439 $\pm$ 0.997	6.480 $\pm$ 0.998	6.498 $\pm$ 0.999	6.509 $\pm$ 1.000	6.515 $\pm$ 1.001	6.517 $\pm$ 1.005	

SD = sampling density; ASD = axial-view sampling density.

Table 3.3: Surface area statistics of (M) measurements from the 3D reconstructed models and (A) analytic calculation from the fitted diameter for calibration particles ($\times 10^5 \mu m^2$).

SD ASD		17.10	10.00	7.94	6.93	6.30	5.85	5.50
4	M	8.171 $\pm$ 0.854	8.353 $\pm$ 0.855	8.396 $\pm$ 0.854	8.423 $\pm$ 0.857	8.442 $\pm$ 0.859	8.446 $\pm$ 0.859	8.443 $\pm$ 0.855
	A	8.102 $\pm$ 0.842	8.226 $\pm$ 0.839	8.247 $\pm$ 0.837	8.258 $\pm$ 0.839	8.266 $\pm$ 0.838	8.269 $\pm$ 0.840	8.269 $\pm$ 0.839
7		7.708 $\pm$ 0.807	7.873 $\pm$ 0.806	7.911 $\pm$ 0.807	7.935 $\pm$ 0.808	7.951 $\pm$ 0.810	7.955 $\pm$ 0.810	7.954 $\pm$ 0.809
		7.705 $\pm$ 0.805	7.842 $\pm$ 0.802	7.869 $\pm$ 0.801	7.882 $\pm$ 0.802	7.889 $\pm$ 0.802	7.893 $\pm$ 0.802	7.895 $\pm$ 0.805
12		7.960 $\pm$ 0.824	8.075 $\pm$ 0.822	8.103 $\pm$ 0.822	8.123 $\pm$ 0.825	8.139 $\pm$ 0.826	8.143 $\pm$ 0.826	8.144 $\pm$ 0.826
		7.925 $\pm$ 0.820	8.028 $\pm$ 0.816	8.041 $\pm$ 0.815	8.049 $\pm$ 0.816	8.053 $\pm$ 0.816	8.055 $\pm$ 0.816	8.056 $\pm$ 0.817
14		7.861 $\pm$ 0.817	7.984 $\pm$ 0.815	8.010 $\pm$ 0.814	8.031 $\pm$ 0.817	8.045 $\pm$ 0.819	8.049 $\pm$ 0.818	8.049 $\pm$ 0.818
		7.865 $\pm$ 0.816	7.967 $\pm$ 0.812	7.983 $\pm$ 0.810	7.992 $\pm$ 0.811	7.996 $\pm$ 0.811	7.999 $\pm$ 0.811	8.000 $\pm$ 0.812
21		7.669 $\pm$ 0.802	7.822 $\pm$ 0.802	7.852 $\pm$ 0.801	7.871$\pm$0.803	7.883 $\pm$ 0.803	7.885 $\pm$ 0.803	7.883 $\pm$ 0.803
		7.673 $\pm$ 0.801	7.810 $\pm$ 0.799	7.832 $\pm$ 0.798	7.843$\pm$0.799	7.849 $\pm$ 0.798	7.852 $\pm$ 0.799	7.852 $\pm$ 0.801
28		7.600 $\pm$ 0.798	7.773 $\pm$ 0.799	7.808 $\pm$ 0.798	7.830 $\pm$ 0.800	7.844 $\pm$ 0.801	7.847 $\pm$ 0.801	7.845 $\pm$ 0.803
		7.606 $\pm$ 0.797	7.764 $\pm$ 0.797	7.796 $\pm$ 0.796	7.810 $\pm$ 0.797	7.820 $\pm$ 0.797	7.825 $\pm$ 0.798	7.826 $\pm$ 0.801
42		7.617 $\pm$ 0.798	7.784 $\pm$ 0.799	7.818 $\pm$ 0.798	7.838 $\pm$ 0.800	7.850 $\pm$ 0.801	7.853 $\pm$ 0.801	7.851 $\pm$ 0.802
		7.622 $\pm$ 0.798	7.776 $\pm$ 0.797	7.806 $\pm$ 0.796	7.819 $\pm$ 0.797	7.827 $\pm$ 0.797	7.832 $\pm$ 0.797	7.833 $\pm$ 0.800
84		7.582 $\pm$ 0.796	7.757 $\pm$ 0.798	7.792 $\pm$ 0.797	7.812 $\pm$ 0.799	7.825 $\pm$ 0.799	7.827 $\pm$ 0.800	7.824 $\pm$ 0.801
		7.588 $\pm$ 0.796	7.750 $\pm$ 0.796	7.782 $\pm$ 0.795	7.797 $\pm$ 0.796	7.806 $\pm$ 0.796	7.811 $\pm$ 0.796	7.812 $\pm$ 0.799

SD = sampling density; ASD = axial-view sampling density.

Table 3.4: Volume statics of zebrafish of the 3 larval stages under study as derived from the 3D reconstructed models ($\times 10^8 \mu m^3$).

ASD dpf	4	7	12	14	21	28	42	84
3	3.60 $\pm$ 0.18	2.67 $\pm$ 0.13	2.64 $\pm$ 0.11	2.60 $\pm$ 0.11	2.53$\pm$0.11	2.52 $\pm$ 0.11	2.49 $\pm$ 0.11	2.46 $\pm$ 0.10
4	3.43 $\pm$ 0.23	2.78 $\pm$ 0.20	2.74 $\pm$ 0.19	2.70 $\pm$ 0.19	2.63$\pm$0.19	2.62 $\pm$ 0.19	2.59 $\pm$ 0.18	2.56 $\pm$ 0.18
5	3.94 $\pm$ 0.23	3.18 $\pm$ 0.19	3.12 $\pm$ 0.18	3.07 $\pm$ 0.18	3.00$\pm$0.18	2.98 $\pm$ 0.18	2.95 $\pm$ 0.18	2.91 $\pm$ 0.18

Table 3.5: Surface area statics of zebrafish of the 3 larval stages under study as derived from the 3D reconstructed models ($\times 10^6 \mu m^2$).

ASD dpf	4	7	12	14	21	28	42	84
3	4.06 $\pm$ 0.17	3.33 $\pm$ 0.16	3.29 $\pm$ 0.18	3.26 $\pm$ 0.16	3.20$\pm$0.15	3.19 $\pm$ 0.15	3.16 $\pm$ 0.14	3.13 $\pm$ 0.14
4	4.29 $\pm$ 0.24	3.48 $\pm$ 0.18	3.44 $\pm$ 0.18	3.40 $\pm$ 0.18	3.34$\pm$0.17	3.32 $\pm$ 0.17	3.29 $\pm$ 0.17	3.26 $\pm$ 0.16
5	4.70 $\pm$ 0.19	3.81 $\pm$ 0.13	3.76 $\pm$ 0.15	3.71 $\pm$ 0.15	3.63$\pm$0.14	3.61 $\pm$ 0.14	3.58 $\pm$ 0.13	3.55 $\pm$ 0.13

ASD = axial-view sampling density; dpf = days post fertilisation.

Table 3.6: Statics of volume ($\times 10^8 \mu m^3$) and surface area ($\times 10^6 \mu m^2$) for living zebrafish larvae

	4 dpf	5 dpf
V	2.69 $\pm$ 0.17	3.01 $\pm$ 0.16
S	3.48 $\pm$ 0.15	3.74 $\pm$ 0.13

V = volume; S = surface area.