ReViMS: Software tool for estimating the volumes of 3-D multicellular spheroids imaged using a light sheet fluorescence microscope

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Cancer 3-D spheroids are widely used to test drugs and radiotherapy treatments. These 3-D cell clusters range from tens to hundreds of micrometers in size, with shapes that typically differ from a perfect sphere. Change in spheroid volume is one of the most important parameters for evaluating treatment efficacy, and using light sheet fluorescence microscopes (LSFM), optical sections of samples in that size range can be obtained. However, there remains a lack of validated methods for quantifying the volumes of 3-D multicellular aggregates. Here, we present Reconstruction and Visualization from Multiple Sections (ReViMS), an open-source, user-friendly software for automatically segmenting z-stacks of fluorescence images and estimating the volumes of 3-D multicellular spheroids. To assess the precision and accuracy of the volume estimates obtained with ReViMS, we used several cancer spheroids imaged with LSFM. Both the precision and accuracy were >95%, demonstrating the effectiveness of ReViMS.

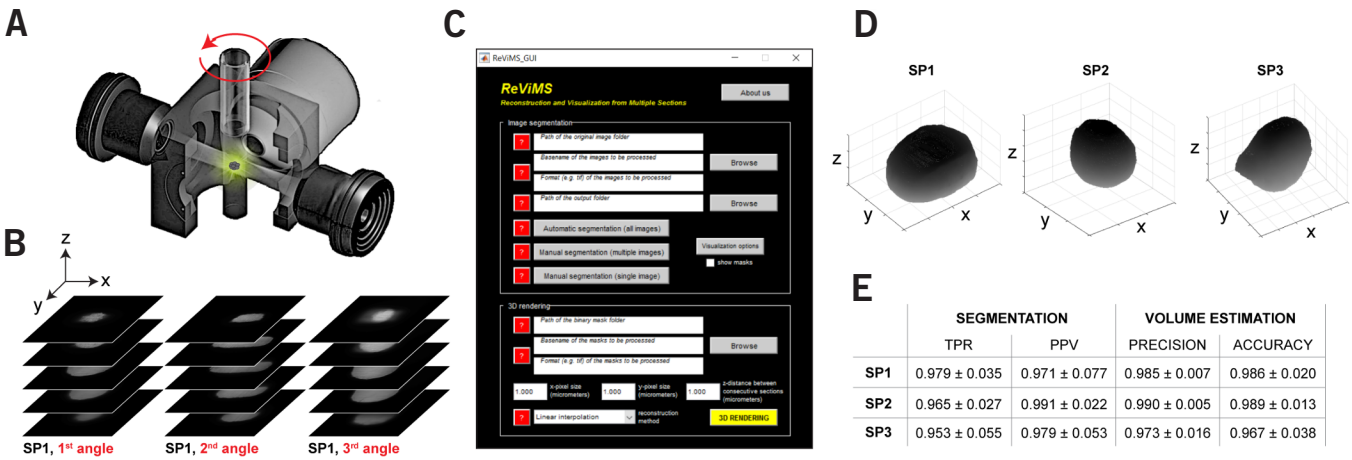
Three-dimensional (3-D) cell culture systems are preferable to two-dimensional (2-D) systems for drug testing because 3-D cell culture better mimics the in vivo tumor (1). The size of cancer spheroids can range from tens to hundreds of micrometers, and their shape, especially after drug treatment, typically differs from a perfect sphere despite the fact that the spheroids are often assumed to be spherical, but this is only a rough approximation (2). Today, the use of cancer spheroids as 3-D in vitro models is an established methodology. Several morphological and biological features of cells in culture can be automatically determined using publicly available software (3). However, the volume of the tumor spheroids before and after treatment—one of their most important characteristics—is challenging to estimate (4).

Confocal and light sheet fluorescence microscopes (LSFM) are typically used for spheroid imaging, since they can provide deep insights into the physiology of the cell aggregates (5). In particular, LSFM can be used to obtain a z-stack of optical sections from a sample by illuminating the specimen with a thin sheet of light. High penetration and low photobleaching/phototoxicity from the LSFM permit analysis of spheroids up to hundreds of micrometers in size. In addition, entire organisms, up to a few millimeters in size (e.g., zebrafish and *Drosophila*) (6), can be analyzed by merging stacks originating from different angles (7). Nevertheless, no validated methods have been reported for accurate estimation of multicellular spheroid volume, and the few tools that are currently available are either not user-friendly or are not freely available (8,9).

Here, we present Reconstruction and Visualization from Multiple Sections (ReViMS), the first validated, open-source, user-friendly software tool for accurately estimating the volume of a spheroid through segmenting the single sections in a z-stack of images of the spheroid and reconstructing its 3-D surface (Figure 1 and Supplementary Video S1). In an earlier report, we presented ReViSP, a software application using a single projection to roughly estimate the volume of a spheroid (4,10). We now extend that study to provide an accurate estimate of the spheroid volume by using a set of sections, in particular a

METHOD SUMMARY

Here, we present Reconstruction and Visualization from Multiple Sections (ReViMS), an open-source, user-friendly software for automatically segmenting z-stacks of fluorescence images and estimating the volume of a 3-D multicellular spheroid. ReViMS provides several tools for segmenting z-stacks of fluorescence images. The 3-D surface area and volume of the spheroid are computed by linearly interpolating the border of the spheroid's sections using the binary masks obtained after the segmentation.



	SEGMENTATION		VOLUME ESTIMATION	
	TPR	PPV	PRECISION	ACCURACY
SP1	0.979 ± 0.035	0.971 ± 0.077	0.985 ± 0.007	0.986 ± 0.020
SP2	0.965 ± 0.027	0.991 ± 0.022	0.990 ± 0.005	0.989 ± 0.013
SP3	0.953 ± 0.055	0.979 ± 0.053	0.973 ± 0.016	0.967 ± 0.038

Figure 1. Reconstruction and Visualization from Multiple Sections (ReViMS) procedure. (A) The Zeiss Lightsheet Z1 microscope allows imaging of the sample from different angles. (B) For each spheroid, we acquired a z-stack from three different angles. (C) The ReViMS graphical user interface (GUI). (D) 3-D surfaces obtained using ReViMS. (E) Segmentation and volume estimation quality (mean ± SD); TPR: true positive rate; PPV: positive predictive value.

z-stack of fluorescence images acquired with a confocal microscope or an LSFM (Supplementary Dataset S1). It is worth noting that this work is the first to validate a method for estimating the volume of a multicellular spheroid imaged using a confocal microscope or an LSFM.

Several methods have been used to generate multicellular spheroids (11). For our experiments, we generated A549 lung cancer spheroids, with a diameter of ~300 µm, using a rotatory RCCS-8DQ bioreactor (Synthecon, Inc., Houston, TX). The spheroids were fixed using a glutaraldehyde solution to obtain an appropriately diffuse and stable autofluorescence (2) and then imaged using a Zeiss Lightsheet Z.1 microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) (Figure 1A). Additional details on spheroid generation and imaging protocols are reported in Supplementary Material Part 1.

ReViMS provides several automatic and manual tools for segmenting z-stacks of fluorescence images (3,12). To analyze spheroids, an automatic method was employed based on a fixed threshold determined by averaging the values obtained according to the Otsu and Triangle histogram segmentation methods (Supplementary Material Part 2). To improve segmentation, vignetting correction (13) and deconvolution (14) can be performed in advance. The 3-D surface and volumes of the spheroids (Figure 1D) were computed by linearly interpolating the border of the spheroid's sections using the binary masks obtained after the segmentation (Supplementary Material Part 3).

Next, to assess the quality of the volume estimations obtained using ReViMS, we imaged several spheroids from 3 different angles, acquiring for each spheroid 3 z-stacks of ~500 sections per stack. The z-stacks of fluorescence images were automatically segmented, and the true positive rate (TPR) and positive predictive value (PPV) were calculated according to equations previously reported by Piccinini (3). As the ground truth, we used the binary masks obtained by an expert operator who manually segmented all of the images in the z-stacks. The (*i*) precision and (*ii*) accuracy of the volume estimates were then determined using ReViMS by computing, respectively, the absolute difference (*i*) among the volumes computed from the different angles and (*ii*) between the single volumes computed from the different angles and the volumes obtained by considering the ground truth masks (Supplementary Table S1). Figure 1E shows the mean ± SD for the values obtained. Both the TPR and PPV as well as the precision and accuracy of the measurements were >95%, demonstrating the effectiveness of ReViMS in computing the volume of a 3-D multicellular spheroid. Finally, we proved that the volumes estimated by ReViMS are reliable in the case of stacks with a low number of z-sections (Supplementary Material Part 4).

ReViMS is an open-source, user-friendly software tool written in MATLAB (The MathWorks, Inc., Natick, MA) and designed for biologists. The source code and standalone software are distributed under the GNU General Public License version 3 and are publicly available for download at *http://sourceforge.net/p/revims*. ReViMS allows reconstruction of the surface of a 3-D spheroid and estimation of its volume without any prior information about the sample or the need for any programming skills. Although similar functionalities are implemented in Amira (Visage Imaging Inc., Carlsbad, CA) and Imaris (Bitplane AG, Zurich, Switzerland) (15), these are commercial tools that are not available to all researchers and require at least basic image-processing skills. To the best of

our knowledge, 3-D ImageJ Suite (16) represents the only freely available solution for segmenting a z-stack of fluorescence images and computing the volumes of imaged objects; however, in addition to not being user-friendly and not having interpolation between different sections, this software has never been validated against a gold-standard method.

ReViMS is the only freely available, user-friendly, and, most importantly, validated solution for segmenting z-stacks of fluorescence images and estimating the volume of a 3-D spheroid. Using different spheroid data sets, we demonstrated that the reliability of the volume data obtained when using ReViMS is comparable with that achievable with Imaris (Supplementary Material Part 5, Supplementary Table S2). In the future, we plan to extend the segmentation methods implemented in ReViMS to process data sets from larger specimens such as zebrafish and *Drosophila*.

Author contributions

F.P. and A.B. conceived and initiated the project. A.T. and M.Z. prepared the samples and collected the data. F.P. and A.B. performed the analysis. F.P., A.T., and M.Z. wrote the manuscript. A.B. revised the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare no competing interests.

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