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Single-image based methods used for non-invasive volume estimation of cancer spheroids: a practical assessing approach based on entry-level equipment

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ABSTRACT

Background: Cancer multicellular spheroids are commonly used as 3D tumour models for testing drugs and radiotherapy treatments. The volume plays a key role in analysis of the results. Several methods have been proposed in the literature to compute the spheroid's volume from one 2D microscopy image (*i.e.* a single projection). However, the literature lacks reviews summarising the different methods available. Furthermore, there are no well-established approaches by which to compare the different methods and determine the best one.

Objective: In this work we (*a*) revise the existing single-image based methods used to estimate the volume of multicellular spheroids, also providing different implementations for classical spherical and ellipsoidal pre-defined models; (*b*) present an upgrade of a volume estimation software recently proposed, *Reconstruction and Visualization from a Single Projection* (*ReViSP*), just validated by using four real spheroids imaged in 3D with a light-sheet microscope; (*c*) propose a quality assessing approach for single-image based methods, relying on 3D home-made macroscopic synthetic models mimicking the shapes of real multicellular spheroids.

Results: Seven image-based methods used to estimate the volume of spheroids were compared using six 3D home-made synthetic models. First, the material used to make the synthetic models was characterised to estimate its density. Then, the ground-truth volume of the 3D models was *measured* by simply weighing them. The volume instances *estimated* by the different methods were compared with ground truth. *ReViSP* attained the best result three times out of six and on average.

Conclusions: The results obtained proved that (*a*) different implementations for the classical spherical and ellipsoidal pre-defined models may lead to very different results; (*b*) *ReViSP* is the best single-image based method available today to estimate the volume of multicellular spheroids.

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1. Introduction

In the literature there are several papers proving that many cell lines behave differently when cultured as monolayer rather than when cultured in 3D [1,2]. In practice, it has been widely established that monolayer cultures are a deficient model. In 1970 Sutherland et al. proposed multicellular aggregates of "spherical" shape, called *spheroids*, as a reliable 3D tumour model grown *in vitro* [3]. These multicellular aggregates morphologically resemble nodules seen in animal and human carcinomas, and this is the reason behind the name spheroids [4]. However, most of the cancer multicellular spheroids grown *in vitro* are not spheres [5]. They are characterised by a local axial rotational symmetry, but often have irregular shapes. Nevertheless, the name spheroid is the one used for these multicellular aggregates, regardless of their real shape.

Today more and more biological laboratories are using cancer spheroids to test drugs and radiotherapy treatments [6,7]. The change in tumour volume is considered as one of the most important features in assessing a treatment [8]. Logically, measuring the volume of a real sphere is trivial, but it becomes complex when the object's shape is irregular. The lack of automatic methods enabling accurate measurement of a spheroid volume strongly limits the reliability of the data obtained [9]. Fluorescent light-sheet microscopy represents the cutting-edge technology [10] to analyse organisms [11] and multicellular spheroids [12]. However, at present, the light-sheet microscope presents several limitations: (*a*) it is a high-cost system not available to most laboratories; (*b*) it works in fluorescence and the stained tissues cannot be used as models for subsequent analysis; (*c*) it does not present validated methods to quantitatively estimate the volume of the object observed. Several, non-invasive, image processing methods have been proposed in the literature to estimate the volume by using a 2D projection of a spheroid, typically a brightfield image (*i.e.* an image obtained without using cytotoxic fluorescent dyes). For instance *Reconstruction and Visualization from a Single Projection* (*ReViSP*), a 3D volume rendering method specifically conceived to estimate the volume of multicellular spheroids, is the latest method presented in the literature [13]. Recently, it has been used to measure the volume of spheroids used in viability [14] and cytotoxic analyses [5]. Of course, assessing the volume from a single image is a challenging task intrinsically prone to error. Nevertheless, the spheroid's volume can be estimated fairly accurately if certain priors are satisfied, such as the axial rotational symmetry.

In this work we (*a*) compare seven single-image based methods for non-invasive estimation of the volume of multicellular spheroids, also considering a very complex-shaped spheroid; (*b*) validate *ReViSP*, using a real multicellular large spheroid imaged through a light-sheet microscope, also providing different implementations for the classical spherical and ellipsoidal rendering models yielding the new release of the software (*i.e. ReViSP v2.0*, http://sourceforge.net/p/revisp); (*c*) describe in details the approach utilised to assess the accuracy of the volume estimation methods, fully characterising the 3D synthetic models and carrying out a proper error analysis of the measurement of the ground-truth volume. In order to determine the ground-truth volume of each synthetic spheroid, a preliminary experiment was performed to estimate the density of the material and the settling time needed to reach a stable asymptotic point. Then, the ground-truth volume of each synthetic spheroid was indirectly achieved by simply using a precision balance. Finally, by processing the 2D images acquired with a digital camera, it was possible to quantitatively compare with ground truth all the volume instances estimated by the different methods.

This article is organised as follows. **Section 1** reports the main aims of the work. **Section 2** presents a short overview of the main single-image based methods used for volume estimation of spheroids. **Section 3** describes the approach proposed to assess the accuracy of the different methods. Experiments performed and results obtained are presented and discussed in **Section 4**. Section 5 summarises the main findings of the work.

2. Volume estimation methods

In this section we describe the methods mostly commonly used to estimate the volume of a multicellular spheroid by analysing a single 2D projection. They are based on different assumptions, but all of them basically exploit the symmetry of the spheroids around their major axis. They require as the input parameter the binary mask (black and white image with 0 for the background and 1 for the foreground) of the widest cross-section of the spheroid to be analysed. In case of spheroids having multi-focus planes, acquiring first several images by scanning the object along the *z*-axis is recommended. Then, a fully focused 2D projection can be reconstructed by automatically merging the different images [15], previously flat-field corrected for radial fall-off attenuation of the image intensity [16]. In the case of spheroids larger than the field of view of the microscope camera, one may manually acquire a set of partially overlapped images that can be automatically stitched into a "mosaic" by using *MicroMos*, a free open-source software [17,18]. Finally, once the image of the spheroid has been obtained, the binary mask can be computed with *AnaSP*, an open source tool specifically designed to segment multicellular spheroids [19].

2.1. *Methods based on a pre-defined geometric model*

The first researchers who considered the problem of estimating the volume of a tumour spheroid by analysing a single 2D projection were Gaylord and Clowes back in 1906 [20]. They used the formula of the *ellipsoid* (**Eq. 1**, where $pi = 3.14159$) to approximate the tumour volume starting from computation of the major axes *M* and the orthogonal one, *P*, passing by the centre of mass:

$$V_{ELLIPSOID} = \frac{pi}{6} \times M \times P^2. \quad (1)$$

In contrast, few years later Woglom [21] proposed using the trivial formula of the *sphere* (**Eq. 2**) by simply computing the equivalent diameter *D*: