



Publication Year	2014
Acceptance in OA @INAF	2023-01-19T11:50:45Z
Title	A New Biometric Tool for Three-Dimensional Subcutaneous Tumor Scanning in Mice
Authors	PISANU, Tonino; BUFFA, Franco; PERNECHELE, Claudio; GUIDO BOCCI; CONCU, Raimondo; et al.
Handle	http://hdl.handle.net/20.500.12386/32931
Journal	IN VIVO
Number	28

A New Biometric Tool for Three-Dimensional Subcutaneous Tumor Scanning in Mice

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Reprinted from

in vivo 28: 75-80 (2014)



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Publication Data: IN VIVO is published bimonthly. Each annual volume comprises six issues. Annual Author and Subject Indexes are included in the sixth issue of each volume. IN VIVO Vol. 18 (2004) and onwards appears online with Stanford University HighWire Press.

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A New Biometric Tool for Three-Dimensional Subcutaneous Tumor Scanning in Mice

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Abstract. *Aim: To propose an innovative methodology for the monitoring of the evolution of induced subcutaneous tumors in mice. Materials and Methods: A new 3D scanner able to measure the tumor mass volume is presented. The scanner is based on the projection of a fringe pattern onto the sample surface (structured light). The lines are diffused by the sample and then collected by a digital camera. The obtained 2D-image is treated by the scanner's software that extracts the 3D information and evaluates the sample volume. Results: The 3D scanner has been successfully used in the measurement of subcutaneous HT-29 colorectal cancer xenografts treated with 5-fluorouracil, bevacizumab and their combination. Comparison with simple caliper measurements revealed important and significant differences between the two measurement techniques. Conclusion: The proposed methodology is more effective than the usual approach based on caliper measurements.*

The efficacy of a new anticancer drug should be always verified by means of the so-called experimental model of the disease, where the characteristics of the disease are recreated in cell cultures, grown in the laboratory (*in vitro* models) or in laboratory animals (*in vivo* models) (1). Considering the latter case, tumors may be induced in mice by subcutaneous injection of human tumor cells. Subcutaneous human tumor xenograft models are widely used because they recapitulate many aspects of the biology of human tumors, including sensitivity to anticancer agents (2). Once cancer cells are inoculated, the researchers carry out treatment with the

anticancer drug and follow the daily evolutions in the shape and in volume of the tumor. The tumor mass volume of treated and non-treated mice must be statistically compared in order to assess the reliability of the treatment. The testing of human tumors in subcutaneous sites have provided relevant and predictive information to the clinic. In fact, every clinically-approved anticancer drug was tested using this model, and showed significant antitumor effects before entering early-phase clinical trials (2).

Although some experimental devices are available for the measurement of tumor volume from 3-dimensional images (computed axial tomography, positron-emission tomography, and magnetic resonance imaging), these systems are primarily designed for biological, chemical, and functional studies of animal models (3). As well-stated by Girit and colleagues, these devices usually require many preparative efforts and expensive materials (radioactive sources, contrast agents, and fluorescent chemicals), thus their use is not practical for animal testing facilities (4).

On the basis of these considerations, in partnership with POEMA (Progettazioni Opto-Elettroniche Metrologia Avanzata; Cagliari, Italy; www.poemaonline.eu), we developed an innovative small non-contact device (3D scanner) capable of rapid, highly reproducible measurements of subcutaneous tumor volumes in mice. We outline the prototype characteristics, describing hardware and software features, and give some practical examples of its use.

Materials and Methods

Project and design. In the project and design phases, we defined a list of requirements and constraints to be imposed on the prototype: i) fast acquisition time and capability to measure volumes in real-time; ii) no mechanical (moving) parts; iii) fully automated-tool with minimal number of manned operations; iv) high spatial resolution; v) easy-to-manage and heavy-duty; vi) low-cost; vii) fast algorithms, robust and reliable code; viii) no need for fast or dedicated computational tools, field programmable gate array (FPGA), digital signal processor (DSP); ix) easy and consistent

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Key Words: Biometry, subcutaneous tumor measurements, 3D scanner, colorectal cancer.

calibration; x) accurate 3D mapping of the sample; xi) easy to use software interface with advanced displaying and editing capabilities; xii) capability to automatically record the outputs in form of spreadsheet format; xiii) capability to save the scanned image files.

Fast acquisition time and real-time measurement mainly drove the design of the 3D scanner; in fact during slow acquisition, the test animal may suddenly change its position, jeopardizing the measurement. We considered several solutions offered by close-range photogrammetry applied to biometry, restricting our attention to *stereo-photogrammetry* and on *structured light* approaches. Both methods, as outlined below, require simple hardware setups and are particularly suited for real-time applications.

Stereo-photogrammetry (or stereo image processing) is based on the retrieval of 3D information from a stereo image pair. Such an approach is largely used in visual processing and in robotics and is based on a very simple setup in which two identical cameras have (mechanically constrained) parallel optical axes. The stereo images are taken simultaneously and 3D information is extracted applying a simplified form of the collinearity equations (5). Stereo image processing may be conceived as a parallel measurement in which the distance, expressed in pixels, from two homologous object points (HOP) is directly related to the Z coordinate in the real world. The method suffers from the difficulty of finding the homologous point pairs, in fact the HOPs are usually sought by means of digital image matching algorithms that use the texture's information to correlate the images. Such an approach lacks reliability when a sample's surface is poorly-textured, as in the case of a mouse's skin. Furthermore, the HOP research algorithms are time-consuming (6) and real-time applications may require for dedicated computational tools to perform the matching.

Structured light is a photogrammetric technique based on the projection of light patterns onto a sample surface. If linear patterns are used, the lines will be deflected by the sample's surface; the stronger the deflection, the greater the distance ΔZ of the object with respect to the reference plane. Figure 1 shows a very simple setup with a telecentric lens system; the object height ΔZ (in pixels) is easily obtained by the equation $\Delta Z = \Delta \phi / \tan \alpha$, where $\Delta \phi$ is the distance (in pixels) between deflected and non-deflected fringes.

Different experimental approaches are possible, for example it is possible to project a set of parallel lines, or instead, a single moving line onto the sample surface; moreover, the projected fringes may be stationary or sliding in one direction (phase-shift) (5). In general, the phase-shift technique may provide better results but suffers from the complexity of ambiguity unwrapping procedures (demodulation) and needs a variable number of images, making its application in real-time difficult. Conversely, amplitude detection of stationary fringes is a very fast approach but is in general coarser in terms of achievable spatial resolution.

As stated before, a single moving line may be projected. In such a case, the line, generally obtained by a laser beam, scans the sample surface by means of a rotating mirror. The acquisition system must be synchronized with the mirror's movements, increasing the hardware complexity. Off-the-shelf models based on single-line approach are available. Because of the mechanical and optical characteristics of such devices, a single scan may take several seconds; we believe that since laboratory animals may move during scanning, a faster method is required.

Taking into account all these factors, we finally designed a prototype based on stationary fringe projection aiming to develop a very fast 3D scanner.

Whatever the hardware setup, the poor optical characteristics of the sample surface require very robust algorithms. In fact, features on mouse skin reflect light irregularly and the skin plicae may (partially) shadow the lines, so that the software must be able to overcome these drawbacks in detecting and fixing any image defects.

The 3D scanner prototype. The 3D scanner prototype comprises three main parts: i) a lighting system projecting a line pattern; ii) a CCD camera with a lens system (1280×1024 pixels); iii) a PC hosting the camera interface, the elaboration software and the user interface.

The projecting system P and the camera C are contained in an aluminum box (300×150×70 mm) forming a constrained angle as shown in Figure 2.

The fringes system f crosses the hole-plate H and is diffused by the sample kept on the opposite side of the reference plane rp. There are many hole-plates with different aperture diameters (ranging from 5 to 30 mm in steps of 5 mm). Each hole-plate is equipped with an easy-to-mount magnet system and may be replaced in a few seconds.

The fringe projection system is composed of three parts, an illumination system, a mask with a printed series of black lines and a focusable optical collimator. The illumination system was a 3 W white LED installed on a C-mount on which the mask and the optical collimator are inserted. The mask is a transparent acetate sheet with a series of black lines 50 μm wide and spaced at 100 μm intervals. The optical collimator is a focusable double Gauss system, which focuses the lines onto the reference plane. The achieved Z resolution is less than 100 μm .

The software. The software manages the handling and synchronization with the CCD camera, and performs the volume calculation. The volume calculation algorithm includes the following: i) image treatment with edge enhancing algorithm; ii) detection and correction of errors (hidden and improperly lit features); iii) identification and measurement of the centroid of the detected lines; iv) identification and correction of false lines; v) volume calculation; vi) saving of the scanned images and the 3D shape files.

It is a well-known fact that camera images may be altered by radial and tangential distortions due to variation in refraction in each individual lens in the objective (5). The software takes into account such distortions, but unlike the standard approach, the corrections are directly applied to the detected lines instead of the whole image.

In our setup, the optical axis of the camera's objective is not orthogonal to the reference plane. This causes perspective anamorphosis, which is a projective distortion altering the 3D image. In practice, the measured shape appears slanted along the Z axis; the software is able to correct such distortions by means of an algorithm derived from the geometry of the optical setup. No further calibrations are needed and no maintenance operations are required by the user.

The graphic user interface (GUI) is used to capture, display and save (for post processing) the 2D image of the sample (Figure 3). The software allows the user to calculate and save the volumes, and to change some parameters to improve the line detection. The results of consecutive volume measurements can be recorded on a spreadsheet for further elaborations. As an option the GUI allows the user to emulate a caliper measurement on the acquired image, which could be useful for the statistical comparisons of the two approaches.

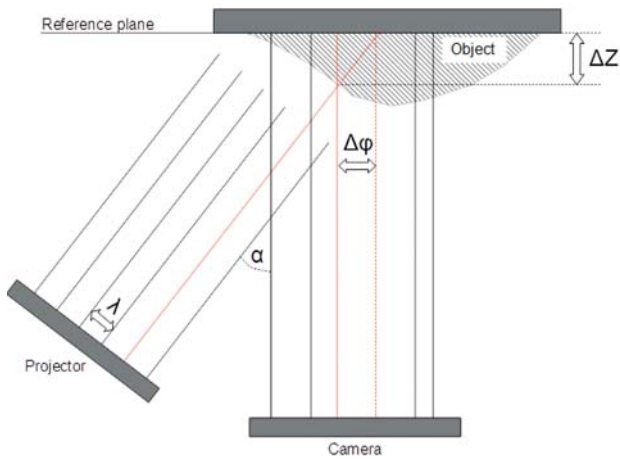


Figure 1. Fringe projection system.

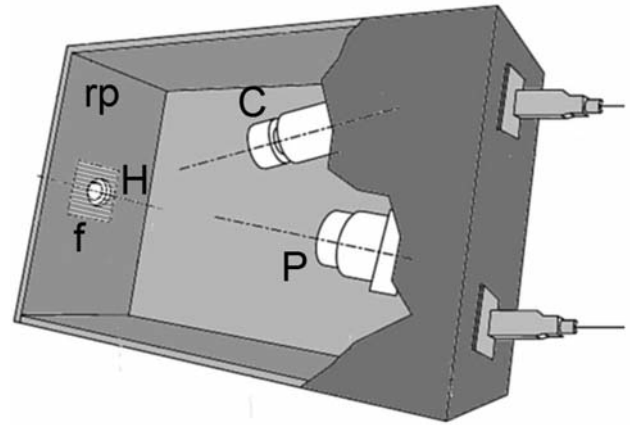


Figure 2. General layout of the device. The pattern f is projected by P onto the sample through the hole H ; the side rp is the image reference plane. The image is recorded by the CCD camera C and sent to the PC.

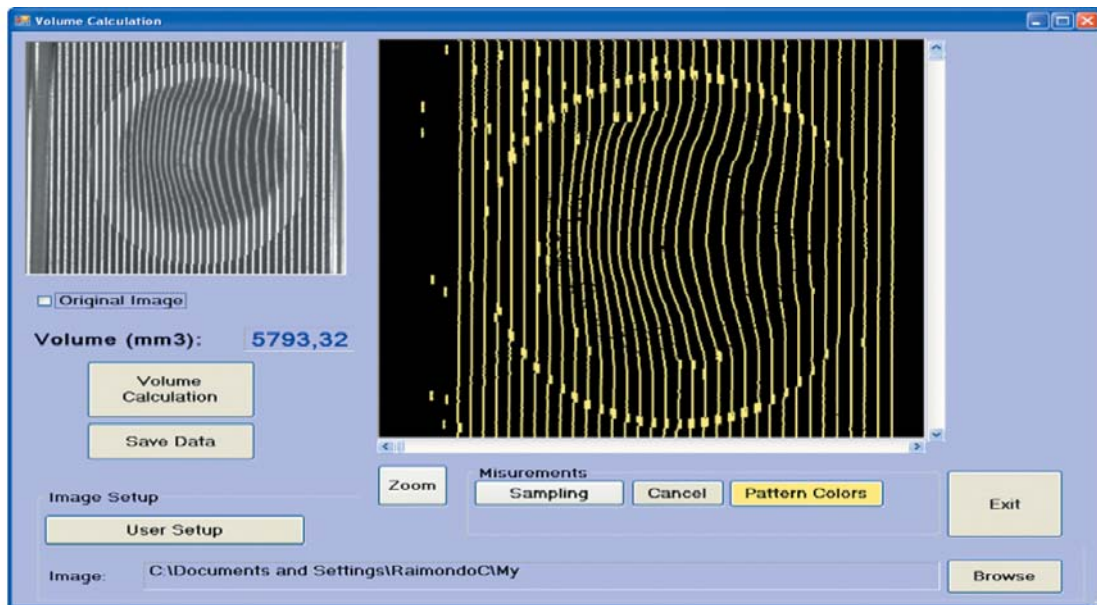


Figure 3. Graphic user interface. The sample is acquired by the CCD camera, the line search procedure is concluded and the volume is calculated and displayed.

An added feature of the software is its capability to save polygon file format (ply) or Stanford triangle format files, allowing study of the sample's morphology in 3D (7). Figure 4 refers to this feature showing the same sample as shown in Figure 3, but represented in 3D.

In vivo experiments

Materials, cell lines and animals. Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Gibco BRL (Paisley, UK), supplements and all other chemicals not listed in this section were obtained from Sigma Chemical Co. (St Louis, MO, USA).

The human colon tumor cell line HT-29 (ATCC, Manassas, MA, USA) was maintained in DMEM supplemented with L-glutamine (2 mM) and 10% Fetal Bovine Serum (FBS).

CD *nu/nu* male mice, weighing 20-25 g, were supplied by Charles River (Milan, Italy) and were allowed unrestricted access to sterile food and water. Housing and all procedures involving animals were performed according to the protocol approved by the Academic Committee for Animal Experimentation of the University of Pisa, in accordance with the European Community Council Directive 86-609, recognised by the Italian government, on animal

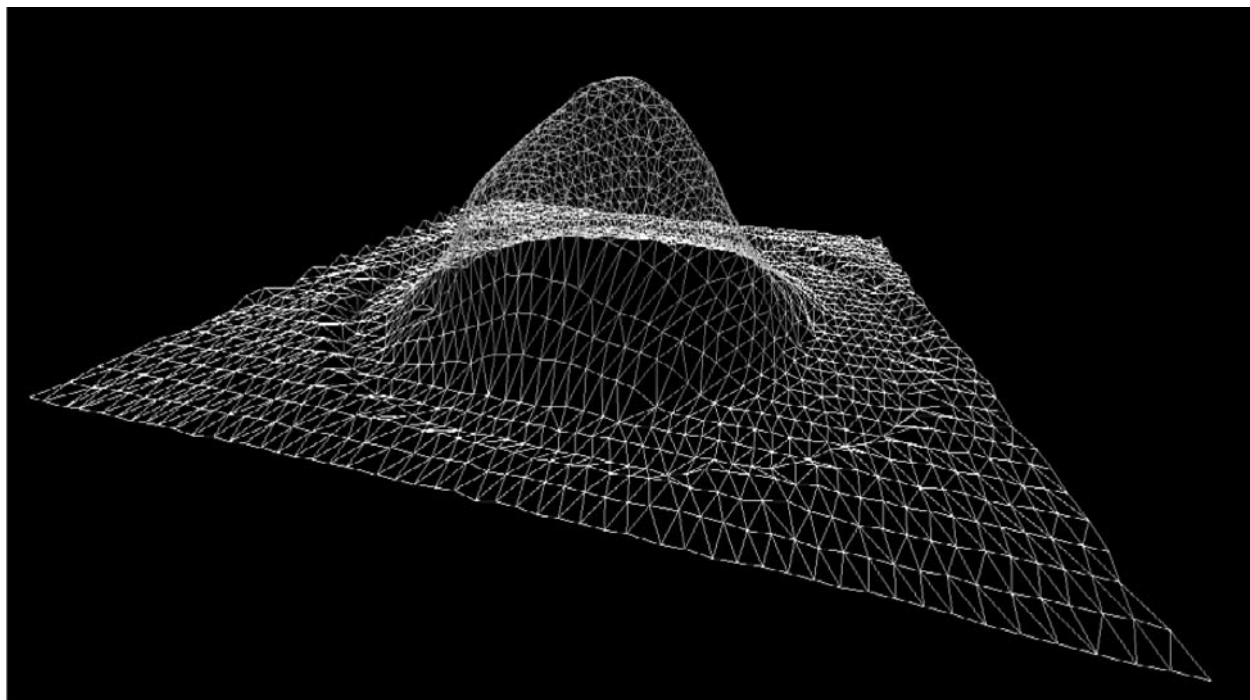


Figure 4. The shape of the sample displayed in Figure 3 as reconstructed by the volume calculation algorithm.

welfare and the guidelines of the UK Co-ordinating Committee on Cancer Research (UKCCCR). Each experiment employed the minimum number of mice needed to obtain statistically meaningful results.

Subcutaneous HT-29 xenografts. HT29 cell viability was assessed by trypan blue dye exclusion, and, on day 0, $1.3 \times 10^6 \pm 5\%$ cells per mouse were inoculated subcutaneously between the scapulae in 0.2 ml of culture medium without FBS using an insulin syringe with a 0.5×16 mm needle (8). Animal weights were monitored and upon the appearance of a subcutaneous mass, tumor dimensions were measured using calipers (by a trained and expert technician) and the 3D scanner. The mice were randomized into groups of eight animals. To treat an established tumor (200 mm^3), from day 20 after cell inoculation, mice were administered *i.p.* bevacizumab (BV) 5 mg/kg every four days (9), 5-fluorouracil (5-FU) 100 mg/kg every four days (10, 11), or a combination of BV and 5-FU. The control group was injected *i.p.* with vehicle alone (saline solution). The experimental period ended by administration of an anesthetic overdose when the control tumor volume reached a mean value of 1000 mm^3 .

Statistical analysis. Analysis of variance followed by the Student-Newman-Keuls test was used to assess the statistical differences in the *in vivo* data among the groups. The *t*-test was used to assess the statistical differences between the group measured with 3D scanner and the caliper. *p*-Values lower than 0.05 were considered significant. Statistical analyses were performed using the GraphPad Prism software package version 5.0 (GraphPad Software Inc., San Diego, CA, USA) (12).

Results

HT-29 cells injected *s.c.* in CD *nu/nu* mice grew quite rapidly, and tumor masses became detectable 10 days after xenotransplantation. Tumors in control animals exhibited progressive enlargement in their dimensions after 20 days; a mean volume of about $1,000 \text{ mm}^3$ was reached at day 38, when the animals of the control group were eliminated (Table I). Both BV and 5-FU inhibited tumor growth and stabilized the tumor masses, although to a different extent; their therapeutic effect was enhanced by their combination (Table I).

As shown in Table I, the 3D scanner was successfully used in the measurement campaign of subcutaneous HT29 xenografts. Both methods described tumor growth similarly. The data obtained from caliper measurements showed higher tumor volumes and standard errors compared to those of 3D scanning (Table I). Indeed, 3D scanning revealed significant differences among the control and treated groups as early as day 29, whereas the caliper did not show any statistical difference among the groups (Table I). Moreover, at day 38, 3D scanning distinguished a statistical difference among the treated groups, whereas the caliper demonstrated only significant differences from the control group. Furthermore, at day 38,

Table I. Measurements of tumor volumes on different days with 3D scanner and a caliper.

Groups	Tumor volumes (mm ³) mean±SEM					
	Day 20		Day 29		Day 38	
	3D Scanner	Caliper	3D Scanner	Caliper	3D Scanner	Caliper
Control	241.9±23.3	287.8±48.8	797.5±54.7	831.5±174.6	1034.9±68.2	1656.9±221.4 [§]
BV	209.7±27.8	244.9±43.1	561.1±50.1*	652.7±178.5	765.6±53.8*	1047.0±245.7*
5-FU	289.0±28.1	324.1±73.6	502.1±42.1*	612.4±125.8	492.0±41.4* [°]	854.8±198.9*
BV+5-FU combination	231.0±21.3	295.0±62.4	418.0±45.1*	507.6±129.5	392.4±43.3* [°]	545.1±102.8*

* $p < 0.05$ vs. control group (ANOVA, Student Newman-Keuls post test); [°] $p < 0.05$ vs. BV group (ANOVA, Student Newman-Keuls post test); [§] $p = 0.017$ 3D scanner vs. caliper (*t*-test analysis). BV, Bevacizumab; 5-FU, 5-fluorouracil.

the mean tumor measurements of the control group were statistically greater when measured with the 3D scanner and the caliper (Table I).

Discussion

Tumor volume (V) measurements are usually performed by means of a caliper, evaluating the largest (W_1) and the smallest (W_2) tumor diameters, applying the formula $V = [(W_1 \times W_2 \times W_2) \times (\pi/6)]$ (8). This experimental approach may be affected by both human errors and formula inadequacy: in fact small tumor masses (a few hundred mm³) are coarsely approximated by a spheroid and this approximation may introduce errors. Moreover, the caliper may deform the soft tissue, depending on the applied pressure, thus introducing more errors. Furthermore, the operator may wrongly report the length and the width of the mass to be measured, introducing further uncertainty. Such errors may strongly affect the results of an experimental campaign.

As a general rule, laboratory practices should be standardized and reproducible (13). Moreover, different operators involved in the same survey may introduce their own bad practices, further degrading the statistical consistency of the measurements. Error must be considered a normal component of human behavior and, for such a reason, highly automated approaches should be preferred when repeatability is a key factor (14).

We show that the caliper method is an approximate and a (relatively) subjective approach, although very skilled personnel were involved. The methodology proposed here based on an innovative 3D scanner was demonstrated in practice to be more effective in finding statistical differences among the groups than the caliper approach, although both measurements were performed by the same experienced researchers.

The 3D Scanning seems to be more reliable and sensitive than calipers being able to describe both disease evolution and drug effects with higher accuracy. The presented results

show that both methods are able to reveal drug efficacy, but the scanner does this significantly earlier. Furthermore, at the end of the experimentation (day 38), the scanner meaningfully depicted the reduction of the tumor volume in 5-FU and BV plus 5-FU cases, while the caliper seemed to lack in accuracy in describing such effects.

Another important issue is the acquisition time: the measurements should be carried out as fast as possible in order to achieve precise measurement and respect animal welfare. The use of caliper is very fast when carried out by trained personnel but may be inaccurate. In this study, we have presented a new tool which can perform very accurate measurements in real time. As an optional feature, the operator may perform the scans as a chained sequence and post-elaborate the data when the survey is ended. This optimizes laboratory practice since the interactions with the computer during the survey are minimized.

Conflicts of Interest

The Authors have no conflict of interest to declare.

Acknowledgements

The Authors thank Dr. Teresa Di Desidero for her precious technical assistance.

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Received September 4, 2013

Revised November 4, 2013

Accepted November 5, 2013

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