ISSN: 0258-851X

in vivo

International Journal of Experimental and Clinical Pathophysiology and Drug Research

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

GUIDO BOCCI[1,3], FRANCO BUFFA[2], BASTIANINA CANU[1], RAIMONDO CONCU[2], ANNA FIORAVANTI[1], PAOLA ORLANDI[1] and TONINO PISANU[2]

[1]*Department of Clinical and Experimental Medicine, University of Pisa, Pisa, Italy;*
[2]*INAF - Astronomical Observatory of Cagliari, Cagliari, Italy;*
[3]*Toscano Tumor Institute, Florence, Italy*

Reprinted from
in vivo *28*: 75-80 (2014)

in vivo

International Journal of Experimental and Clinical Pathophysiology and Drug Research

ISSN (print): 0258-851X, ISSN (online): 1791-7549

Editorial Office: International Institute of Anticancer Research, 1st km Kapandritiou-Kalamou Rd., Kapandriti, P.O. Box 22, Attiki 19014, Greece. Tel / Fax: +30-22950-53389. e-mail: journals@iiar-anticancer.org

General Policy: IN VIVO is a multidisciplinary journal designed to bring together original high quality works and reviews on experimental and clinical biomedical research within the framework of comparative physiology and pathology. The special focus of the journal is the publication of works on: (a) experimental development and application of new diagnostic procedures; (b) pharmacological and toxicological evaluation of new drugs and drug combinations; (c) development and characterization of models for biomedical research.

IN VIVO supports: (a) the activities of the INTERNATIONAL INSTITUTE OF ANTICANCER RESEARCH (IIAR; Kapandriti, Attiki, Greece) and (b) the organization of the International Conferences of Anticancer Research (www.iiar-anticancer.org).

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.

Annual Subscription Rates 2014: Institutional, Euro 855.00 - print or online; Personal, Euro 452.00 - print or online. Prices include rapid delivery and insurance. Previous volumes of IN VIVO (Vol. 1-27, 1987-2013) are available at 50% discount on the above rates.

Subscription Orders: Orders can be placed at agencies, bookstores, or directly with the Publisher. Cheques should be made payable to Delinasios G. J. & CO G.P., Athens, Greece.

Articles in IN VIVO are regulary indexed in bibliographic services, including Index Medicus, PubMed, MEDLINE, Biological Abstracts, Chemical Abstracts, BIOSIS, Chemical Abstracts, Excerpta Medica, Elsevier Bibliographic Database, EMBASE, Compendex, GEOBASE, EMBiology, Elsevier BIOBASE, FLUIDEX, World Textiles, Scopus, CANCER-LIT Database, University of Sheffield Biomedical Information Service (SUBIS), Current Clinical Cancer, AIDS Abstracts, Progress in Paliative Care, Update-Research Information Systems Inc., Inpharma-Reactions Datastar, BRS), Reference Update (I.S.I.), Research Alert, Science Citation Index Expanded, Biochemistry & Biophysics Citation (I.S.I.), BioBase, MedBase, Google Scholar, Investigational Drugs Database, VINITI Abstracts Journal, PubsHub, SIIC Data Bases.

The Editors and Publishers of IN VIVO accept no responsibility for the opinions expressed by the contributors or for the content of advertisements appearing therein.

All correspondence (subscription orders, reprint orders, status of submitted manuscripts, change of address, general editorial matters, advertising rate requests) should be addressed to the Editorial Office. e-mail: journals@iiar-anticancer.org

PRINTED ON ACID-FREE PAPER

D.T.P. BY IIAR
PRINTED BY ENTYPO - ATHENS - GREECE

in vivo *28*: 75-80 (2014)

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

GUIDO BOCCI[1,3], FRANCO BUFFA[2], BASTIANINA CANU[1], RAIMONDO CONCU[2], ANNA FIORAVANTI[1], PAOLA ORLANDI[1] and TONINO PISANU[2]

[1]*Department of Clinical and Experimental Medicine, University of Pisa, Pisa, Italy;*
[2]*INAF - Astronomical Observatory of Cagliari, Cagliari, Italy;*
[3]*Toscano Tumor Institute, Florence, Italy*

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

Correspondence to: Guido Bocci, MD, Ph.D., Division of Pharmacology, Department of Clinical and Experimental Medicine, University of Pisa, Via Roma 55, 56126 Pisa, Italy. Tel: +39 0502218756, Fax: +39 0502218758, e-mail: guido.bocci@med.unipi.it

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 biometrology, 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 paralled measurement in which the distance, expressed in pixels, from two homologous object points (HOP) is directly related to the Z coordinate in the real word. 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.

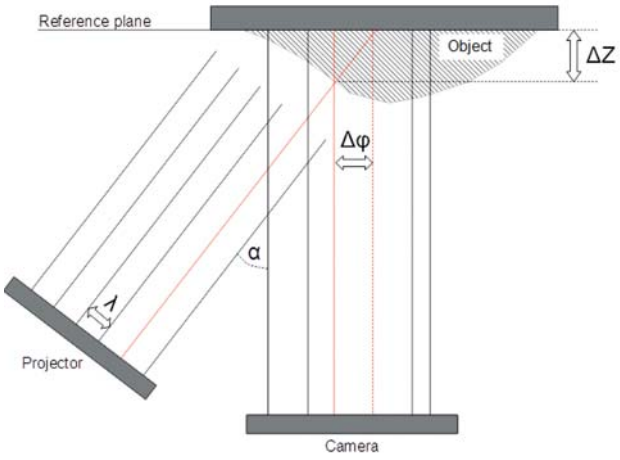

Figure 1. *Fringe projection system.*

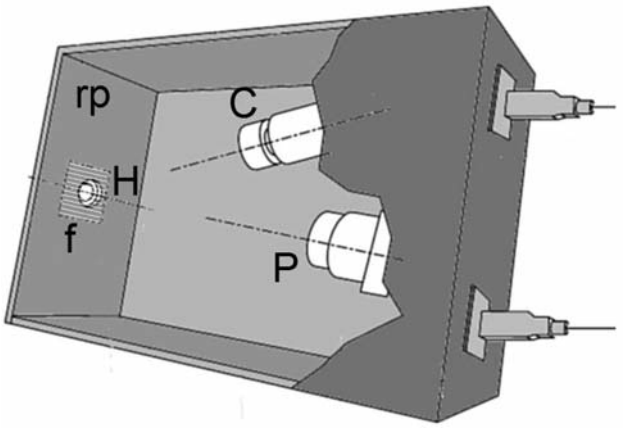

Figure 2. *General layout of the device. The pattern f is projected by P onto the sample through the hole H; the side rp is the image reference plane. The image is recorded by the CCD camera C and sent to the PC.*

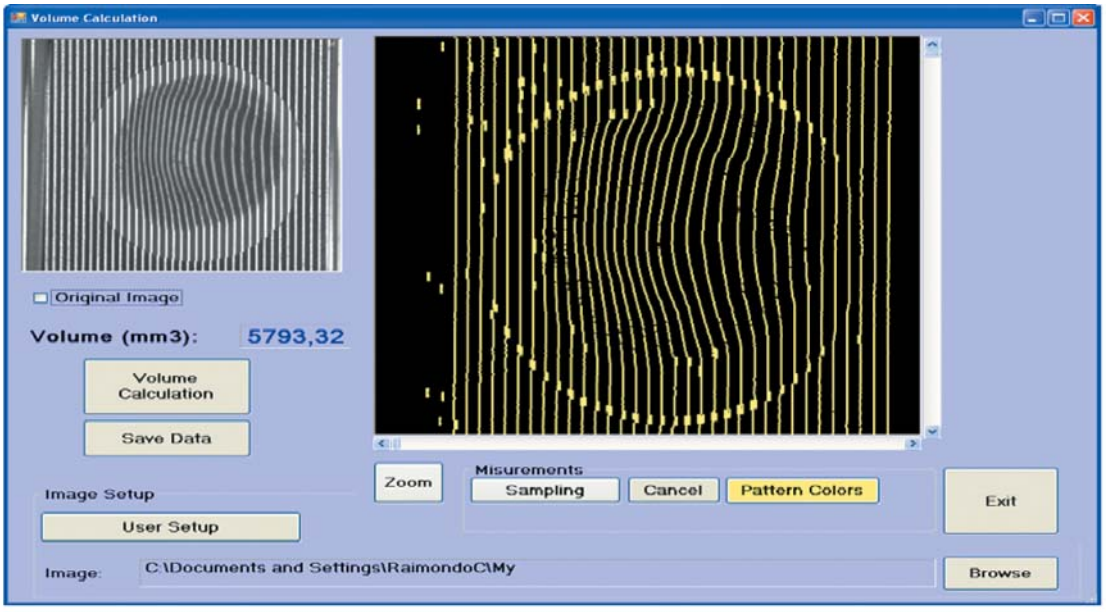

Figure 3. *Graphic user interface. The sample is acquired by the CCD camera, the line search procedure is concluded and the volume is calculated and displayed.*

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

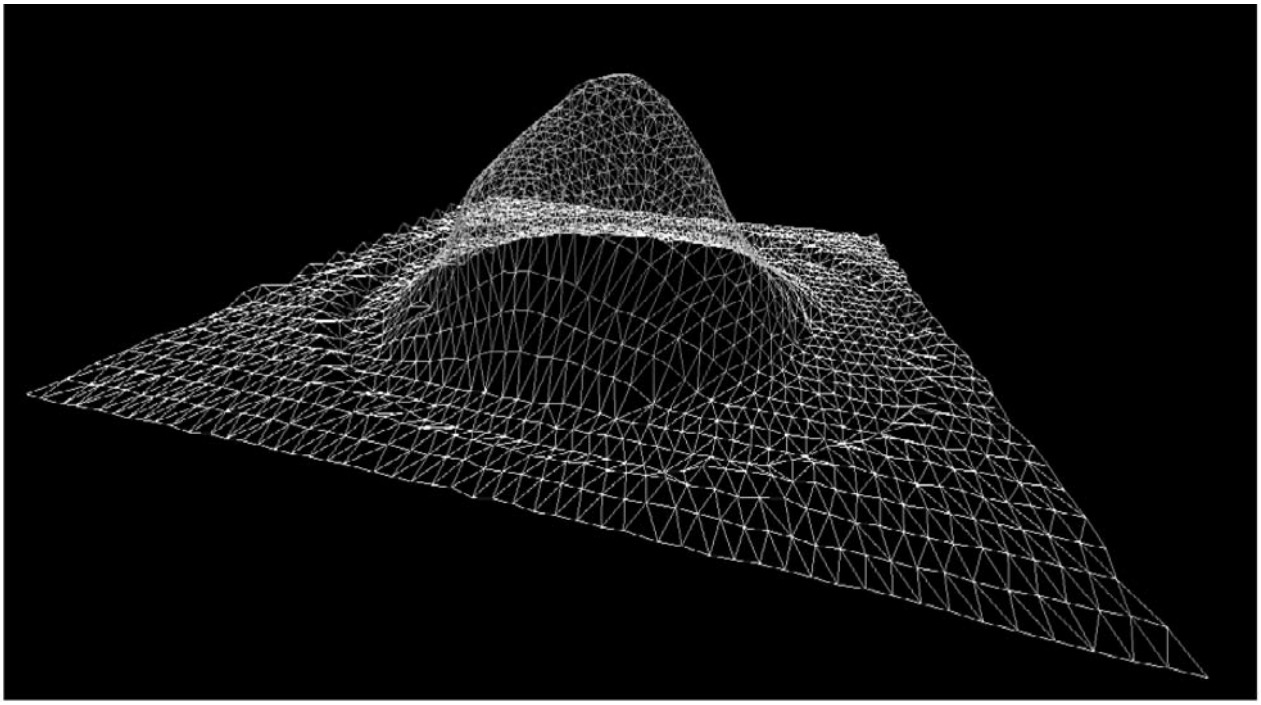

Figure 4. *The shape of the sample displayed in Figure 3 as reconstructed by the volume calculation algorithm.*

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, 1.3×106±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 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 mm^3) 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 where 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.

References

1 Workman P, Aboagye EO, Balkwill F, Balmain A, Bruder G, Chaplin DJ, Double JA, Everitt J, Farningham DA, Glennie MJ, Kelland LR, Robinson V, Stratford IJ, Tozer GM, Watson S, Wedge SR and Eccles SA, Committee of the National Cancer Research Institute: Guidelines for the welfare and use of animals in cancer research. Br J Cancer *102*: 1555-1577, 2010.

2 Kerbel RS: Human tumor xenografts as predictive preclinical models for anticancer drug activity in humans: Better than commonly perceived-but they can be improved. Cancer Biol Ther *2(4 Suppl 1)*: S134-S139, 2003.

3 O'Neill K, Lyons SK, Gallagher WM, Curran KM and Byrne AT: Bioluminescent imaging: a critical tool in pre-clinical oncology research. J Pathol *220*: 317-327, 2010.
4 Girit IC, Jure-Kunkel M and McIntyre KW: A structured light-based system for scanning subcutaneous tumors in laboratory. Compar Med *58*: 264-270, 2008.
5 Luhmann T, Robson S, Kyle S and Harley I: Close Range Photogrammetry: Principles, Methods and Applications. Whittles Publishing, UK; Cdr edition (July 1, 2011).
6 Bae K-H, Ko J-H and Lee J-S: Stereo image reconstruction using regularized adaptive disparity estimation. J Electron Imaging *16(1)*: 013013, 2007.
7 Bradski G and Kaehler A: Computer Vision in C++ with the OpenCV Library. Learning OpenCV, 2nd Edition. O'Reilly Media Released Publisher. October 2012.
8 Bocci G, Falcone A, Fioravanti A, Orlandi P, Di Paolo A, Fanelli G, Viacava P, Naccarato AG, Kerbel RS, Danesi R, Del Tacca M and Allegrini G: Antiangiogenic and anticolorectal cancer effects of metronomic irinotecan chemotherapy alone and in combination with semaxinib. Br J Cancer *98*: 1619-1629, 2008.
9 Zhen Z, Sun X, He Y, Cai Y, Wang J and Guan Z: The sequence of drug administration influences the antitumor effects of bevacizumab and cyclophosphamide in a neuroblastoma model. Med Oncol *28(Suppl 1)*: S619-S625, 2011.
10 Fioravanti A, Canu B, Alì G, Orlandi P, Allegrini G, Di Desidero T, Emmenegger U, Fontanini G, Danesi R, Del Tacca M, Falcone A and Bocci G: Metronomic 5-fluorouracil, oxaliplatin and irinotecan in colorectal cancer. Eur J Pharmacol *619(1-3)*: 8-14, 2009.
11 Azrak RG, Cao S, Slocum HK, Toth K, Durrani FA, Yin MB, Pendyala L, Zhang W, McLeod HL and Rustum YM: Therapeutic synergy between irinotecan and 5-fluorouracil against human tumor xenografts. Clin Cancer Res *10*: 1121-1129, 2004.
12 Di Desidero T, Fioravanti A, Orlandi P, Canu B, Giannini R, Borrelli N, Man S, Xu P, Fontanini G, Basolo F, Kerbel RS, Francia G, Danesi R and Bocci G: Antiproliferative and proapoptotic activity of sunitinib on endothelial and anaplastic thyroid cancer cells *via* inhibition of Akt and ERK1/2 phosphorylation and by down-regulation of cyclin-D1. J Clin Endocrinol Metab *98*: E1465-E1473, 2013.
13 Vesper HW and Thienpont LM: Traceability in laboratory medicine. Clin Chem *55*: 1067-1075, 2009.
14 Joint Committee for Guides in Metrology (JCGM). Evaluation of measurement data: guide to the expression of uncertainty in measurement. Sèvres, France: JCGM, 2008.

Received September 4, 2013
Revised November 4, 2013
Accepted November 5, 2013

Instructions to Authors 2014

General Policy. IN VIVO is a multidisciplinary journal designed to bring together original high quality works and reviews on experimental and clinical biomedical research. The principal aim of IN VIVO is to provide prompt (print and online) publication for accepted articles, generally within 1-2 months from final acceptance.

Manuscripts will be accepted on the understanding that they report original unpublished works that are not under consideration for publication by another journal, and that they will not be published again in the same form. All authors should sign a submission letter confirming the approval of their article contents. All material submitted to IN VIVO will be subject to review, when appropriate, by two members of the Editorial Board. The Editors reserve the right to improve manuscripts on grammar and style.

The use of animals in biomedical research should take place under careful supervision of a person adequately trained in this field and the animals must be treated humanely at all times. Such research should adhere to the Guiding Principles in the Care and Use of Animals approved by the Council of the American Physiological Society.

The Editors and Publishers of IN VIVO accept no responsibility for the contents and opinions expressed by the contributors. Authors should warrantee due diligence in the creation and issuance of their work.

NIH Open Access Policy. The journal acknowledges that authors of NIH funded research retain the right to provide a copy of the final manuscript to the NIH four months after publication in IN VIVO, for public archiving in PubMed Central.

Copyright. Once a manuscript has been published in IN VIVO, which is a copyrighted publication, the legal ownership of all published parts of the paper has been transferred from the Author(s) to the journal. Material published in the journal may not be reproduced or published elsewhere without written consent of the Managing Editor or Publisher.

Format. Two types of papers may be submitted: (i) Full papers containing completed original work, and (ii) review articles concerning fields of recognisable progress. Papers should contain all essential data in order to make the presentation clear. Reasonable economy should be exercised with respect to the number of tables and illustrations used. Papers should be written in clear, concise English. Spelling should follow that given in the "Shorter Oxford English Dictionary".

Manuscripts. Submitted manuscripts should not exceed fourteen (14) pages (approximately 250 words per double - spaced typed page), including abstract, text, tables, figures, and references (corresponding to 4 printed pages). Papers exceeding four printed pages will be subject to excess page charges. All manuscripts should be divided into the following sections: (a) *First page* including the title of the presented work [not exceeding fifteen (15) words], full names and full postal addresses of all Authors, name of the Author to whom proofs are to be sent, key words, an abbreviated running title, an indication " review" , "clinical", "epidemiological", or "experimental" study, and the date of submission. (Note: The order of the Authors is not necessarily indicative of their contribution to the work. Authors may note their individual contribution(s) in the appropriate section(s) of the presented work); (b) *Abstract* not exceeding 150 words, organized according to the following headings: Background/Aim - Materials and Methods/Patients and Methods - Results - Conclusion; (c) *Introduction;* (d) *Materials and Methods/Patients and Methods;* (e) *Results;* (f) *Discussion;* (g) *Acknowledgements;* (h) *References.* All pages must be numbered consecutively. Footnotes should be avoided. Review articles may follow a different style according to the subject matter and the Author's opinion. Review articles should not exceed 35 pages (approximately 250 words per double-spaced typed page) including all tables, figures, and references.

Figures. All figures (whether photographs or graphs) should be clear, high contrast, at the size they are to appear in the journal: 8.00 cm (3.15 in.) wide for a single column; 17.00 cm (6.70 in.) for a double column; maximum height: 20.00 cm (7.87 in.). Graphs must be submitted as photographs made from drawings and must not require any artwork, typesetting, or size modifications. Symbols, numbering and lettering should be clearly legible. The number and top of each figure must be indicated. Colour plates are charged.

Tables. Each table should be submitted on a separate page, typed double-spaced. Tables should be numbered with Roman numerals and should include a short title.

Nomenclature and Abbreviations. Nomenclature should follow that given in "Chemical Abstracts", "Index Medicus", "Merck Index", "IUPAC – IUB", "Bergey' s Manual of Determinative Bacteriology", The CBE Manual for Authors, Editors and Publishers (6th edition, 1994), and MIAME Standard for Microarray Data. Human gene symbols may be obtained from the HUGO Gene Nomenclature Committee (HGNC) (http://www.gene.ucl.ac.uk/). Approved mouse nomenclature may be obtained from http://www.informatics.jax.org/. Standard abbreviations are preferable. If a new abbreviation is used, it must be defined on first usage.

References. Authors must assume responsibility for the accurancy of the references used. Citations for the reference sections of submitted works should follow the standard form of "Index Medicus" and must be numbered consecutively. In the text, references should be cited by number. Examples: 1 Sumner AT: The nature of chromosome bands and their significance for cancer research. Anticancer Res 1: 205-216, 1981. 2 McGuire WL and Chamnes GC: Studies on the oestrogen receptor in breast cancer. In: Receptors for Reproductive Hormones (O' Malley BW, Chamnes GC (eds.). New York, Plenum Publ Corp., pp 113-136, 1973.

Clinical Trials. Authors of manuscripts describing clinical trials should provide the appropriate clinical trial number in the correct format in the text.

For International Standard Randomised Controlled Trials (ISRCTN) Registry (a not-for-profit organization whose registry is administered by Current Controlled Trials Ltd.) the unique number must be provided in this format: ISRCTNXXXXXXXX (where XXXXXXXX represents the unique number, always prefixed by " ISRCTN"). Please note that there is no space between the prefix " ISRCTN" and the number. Example: ISRCTN47956475.

For Clinicaltrials.gov registered trials, the unique number must be provided in this format: NCTXXXXXXXX (where XXXXXXXX represents the unique number, always prefixed by " NCT"). Please note that there is no space between the prefix " NCT" and the number. Example: NCT00001789.

Ethical Policies and Standards. IN VIVO agrees with and follows the "Uniform Requirements for Manuscripts Submitted to Biomedical Journals" established by the International Committee of Medical Journal Editors in 1978 and updated in October 2001 (www.icmje.org). Microarray data analysis should comply with the "Minimum Information About Microarray Experiments (MIAME) standard". Specific guidelines are provided at the "Microarray Gene Expression Data Society" (MGED) website. Presentation of genome sequences should follow the guidelines of the NHGRI Policy on Release of Human Genomic Sequence Data. Research involving human beings must adhere to the principles of the Declaration of Helsinki and Title 45, U.S. Code of Federal Regulations, Part 46, Protection of Human Subjects, effective December 13, 2001. Research involving animals must adhere to the Guiding Principles in the Care and Use of Animals approved by the Council of the American Physiological Society. The use of animals in biomedical research should be under the careful supervision of a person adequately trained in this field and the animals must be treated humanely at all times. Research involving the use of human foetuses, foetal tissue, embryos and embryonic cells should adhere to the U.S. Public Law 103-41, effective December 13, 2001.

Submission of Manuscripts. Please follow the Instructions to Authors regarding the format of your manuscript and references. There are 3 ways to submit your article (NOTE: Please use only one of the 3 options. Do not send your article twice.):

1. To submit your article online please visit: IIAR-Submissions (link to: http://www.iiar-anticancer.org/submissions/login.php)
2. You can send your article via e-mail to journals@iiar-anticancer.org (mail to: journals@iiar-anticancer.org). Please remember to always indicate the name of the journal you wish to submit your paper. The text should be sent as a Word document (*doc) attachment. Tables, figures and cover letter can also be sent as e-mail attachments.
3. You can send the manuscript of your article via regular mail in a USB stick, DVD, CD or floppy disk (including text, tables and figures) together with three hard copies to the following address:
   John G. Delinasios, International Institute of Anticancer Research (IIAR), Editorial Office of ANTICANCER RESEARCH,
   IN VIVO and CANCER GENOMICS & PROTEOMICS, 1st km Kapandritiou-Kalamou Road, P.O. Box 22, GR-19014 Kapandriti, Attiki, GREECE.

Submitted articles will not be returned to Authors upon rejection.

Galley Proofs. Unless otherwise indicated, galley proofs will be sent to the first-named Author of the submission. Corrections of galley proofs should be limited to typographical errors. Reprints, PDF files, and/or Open Access may be ordered after the acceptance of the paper. Requests should be addressed to the Editorial Office.