Cell volume and geometric parameters determination in living cells using confocal microscopy and 3D reconstruction

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Abstract

The protocol reported here describes a simple, easy, fast and reproducible method aimed to know the geometric parameters of living cells based on confocal laser scanning microscopy combined with 3D reconstruction software. Briefly, the method is based on intrinsic fluorescence properties of acridine orange \(AO), a molecule taken up by living adherent cells. Dual binding of AO to either DNA or RNA allows complete staining. When combined with confocal microscopy, 3D software can be used for in vivo living cell reconstruction. Beside the purpose that we intend here, a fast and easy system for cell volume determination, the protocol is an easy approach to study changes in morphology during cellular processes such as cell differentiation. Novel therapeutic approaches would require some knowledge about how these drugs enter into cells/tissues. For this purpose fast and accurate in vivo cell volume determinations such as the method reported here, in combination with analytical methods, would allow estimating intracellular concentrations of compounds and might be further employed for finding out whether any new drug can reach the effective concentration inside its cellular target. Furthermore this protocol with minimal adjustments will permit the determination of morphometric parameters in vivo in different types of adherent cells.

Introduction

It is widely accepted that confocal laser scanning microscopy \(CLSM) and other derivative techniques \(e.g. FRET) are currently the state-of-the-art image technologies in microscopy. Because of the top quality and high definition of cell images provided, CLSM is therefore used for many applications including immunocytochemical detection, nuclear or other organelle localization of target proteins, FRET or any other fluorophore-based technique for different purposes in which high resolution images of cells are required, even in living cells and tissues[1,2]. Nonetheless in addition to its cell image capabilities, this powerful technique has opened a broad range of new image-related possibilities to solve biological questions[3]. Therefore confocal microscopy is a great tool in order to obtain three-dimensional object images using two-dimensional optical sectioning plus 3D reconstruction[4]. For this purpose, image processing software has evolved at such a high speed during the last few years that it has made possible to manage, combine and process digital images in a very short time. Accurate cell volume determination is extremely useful for many Cell Biology techniques, including morphometric studies, physiological studies[5] or estimation of intracellular concentration of substances[6]. Interestingly there are not many simple and straightforward methodological approaches for the estimation of volume especially in living cells. Furthermore many of those methods often requires very specific technology[7], use of isotopes[8] or non-friendly software that requires a time-consuming, long-learning curve. This sometimes discourages users from employing such methods for morphometric analysis. As we suggest here, internalization of targeted drugs could be easily monitored and quantified using both a simple protocol for volume determination as well as parallel analytical methods for quantification of substances inside cells. Therefore combination of these powerful techniques, confocal microscopy and HPLC, which are available for most of laboratories, is the key for these types of pharmacokinetic studies. With this goal in mind, the

aim was to develop an easy protocol to obtain cell images for volume determination but maintaining them in their own growth media so osmotic pressure and therefore potential morphological changes are minimized. Consequently cell volume estimated under this paradigm is more realistic since cell manipulation is minimized. **ADVANTAGES AND DISADVANTAGES OF OUR METHOD** Flow cytometry can also be employed to determine with accuracy cell volume using fluorescent latex particles with a known diameter as standards[9]. Even though this method appears as a good choice in some cases, cells processed for flow cytometry are submitted to some handling prior to injection \(i.e. centrifugation) which can alter the normal volume that they exhibit in cell culture plates, so real values may differ. Furthermore prior to flow cytometer analysis cells are commonly detached from substrate and centrifuged which always induces round shape morphology with changes in real cell volume. However, the method we propose here, using CLSM combined with 3D image processing software and cell cultures, is very simple and fast for measuring geometric parameters of cells and would open new possibilities of knowledge.

Reagents

REAGENTS NaCl \(Sigma, Cat. N°. S3014) KCl \(Sigma, Cat. N°. P4504) Na_2HPO_4 \(Sigma, Cat. N°. S5136) KH_2PO_4\(Sigma , Cat. N°. P5655) HCl \(Sigma, Cat. N°. H1758) HEPES \(Sigma, Cat. N°. H4034) Ultraglutamine 1, 200 mM in 0,85% NaCl solution \(Lonza, Cat. N°. BE17-605E/U1) Antibiotic-antimycotic 100x \(GIBCO, Invitrogen, Cat. N°. 15240) Ampicillin sodium salt \(SIGMA- ALDRICH, Cat. N°. A9518-56) Kanamycin B sulfate salt \(Sigma, Cat. N°. B5264-250M6) Trypsin, 0.25% \(1X) with EDTA \(Invitrogen, Cat. N°. 25200-072) Fetal Bovine Serum E.C. Approved \(GIBCO, Invitrogen, Cat. N°. 10106) Acridine Orange \(Calbiochem, MERK, Cat. N°. 113000-1GM) Mounting medium for microscope preparation EUKITT \(Aname, Cat. N°. RT15320) Inmersion liquid, type F \(Leica microsystem, GmbH, Cat. N°. 11513859 **Cell culture** Corning 75 cm2 Flask, Canted Neck \(BD Biosciences, Cat. N°. H108CO2836) Cell Culture Plate, 6-well, \(BD Falcon™, Cat. N°. 353046) Cell Culture Coverslip, Sterile, Thermanox plastic, 13 mm diameter \(NUNC™ Brand Products, Cat. N°. 174950) Microscope Slides, 76 x 26 mm \(Menzel-Gläser, Cat. N°. AA00000112E) Microscope Cover Slips, 24 x 36 mm \(Menzel-Gläser, Cat. N°. BB024036A1) Counting Chamber Improved Neubauer \(BRAND, Cat. N°. 717805) Sterile Pipette 10 ml \(APL, Cat. N°. PN10E1), 1 ml \(APL, Cat. N°. PN1E1) Micropipette PIPETMAN P1000 \(Gilson, Cat. N°. F123602), P100 \(Gilson, Cat. N°. F123615), P10 \(Gilson, Cat. N°. F144802) Syringe Filter, Acrodisc \(PAAL corporation , Cat. N°. PN 4433) Syringe 5ml, luer slip \(BD Discardit , Cat. N°. 309050) Universal yellow tip 5-200 µl, blue tip 100-1000 µl, tip 1-5 ml \(Daslab, Cat. N°. 162001, 162222, 162005) and tip 0,1-10 µl \(Deltalab, Cat. N°. 200024) Microtubes MCT-150-C 1,5 ml \(Axygen Quality, Cat. N°. 311-08-051) Conical tube 15 ml \(17 x 120 mm) \(BD Falcon, Cat. N°. 352096) Conical tube 50 ml \(30 x 115 mm) \(BD Falcon, Cat. N°. 352070) Pasteur Pipettes - soda glass \(230mm) \(Deltalab, Cat. N°. 702) Manual Counter cash. 4 digits \(Quirumed, Cat. N°. 052-63047001) Tweezers, style 62A \(ANAME, Cat. N°. 78350-62A) **REAGENTS SETUP** Ampicillin stock 500x: Dissolve 500 mg of ampicillin powder in milli-Q H2O to a final volume of 100 ml. Filter-sterilized and store 1 ml aliquots at - 20°C. Kanamicyn stock 500x: Dissolve 500 mg of kanamicyn powder in milli-Q H2O to a final volume of 100 ml. Filter-sterilized and store 1 ml aliquots at -20°C HEPES stock 100x: Dissolve 26.029 g of HEPES and adjust to a final volume

of 500 ml with milli-Q H_2O and filter sterilize. Store at 4°C. Acridine Orange solution: Dissolve 20 mg of acridine orange in 20 ml of PBS and then filter it and keep in the dark to avoid light. Before using, dilute it until 40 µg/ml in PBS. In order to obtain final concentration 10 µl of this solution is added to cultured medium. Phosphate saline buffer \(PBS): Dissolve 0.2 g of KCl , 0.2 g of KH_2PO_4, 8 g of NaCl , 1.15 g of Na_2HPO_4 in 1 L of milli-Q H_2O and filter sterilize. **Cell lines** Mouse embryonic fibroblast cell line \(NIH3T3) \(ATCC, CRL-1658) Normal human prostate epithelium immortalized with SV40 cells \(PNT1A) \(Sigma 95012614) Chinese hamster ovary cell line \(CHO) \(ATCC, CCL-61) Androgen-sensitive human prostate adenocarcinoma cell line \(LNCaP) \(Sigma-Aldrich, 89110211) Human prostate carcinoma cell line \(DU 145) \(ATCC , HTB-81) Androgen-insensitive human adenocarcinoma cell line \(PC3) \(ATCC, CRL-1435) Human breast adenocarcinoma cells \(MCF7) \(ATCC, HTB-22) Human epithelial cervical carcinoma cell line \(HeLa) \(Sigma Aldrich, 93021013) Murine melanoma cell line \(B16-F10) \(ATCC, CRL-6475) Mouse leukemic monocyte macrophage cell line \(RAW 264.7) \(ATCC , TIB-71) Rat glioma cell line \(C6) \(ATCC, CCL-107). **Growth media and supplements** RPMI 1640 \(Lonza, Cat. N°.BE12-167F sterile filtered) supplemented with 10% Fetal Bovine Serum \(FBS), 2 mM Ultraglutamine, 0,1% Ampicillin, 0,1% kanamycin and 15 mM HEPES. DMEM \(Dulbecco´s Modified Eagle´s Medium with 1 g/L Glucose, without L-Glutamine)\(Lonza, Cat. N°.BER-707F) supplemented with 10% Fetal Bovine Serum \(FBS), 2 mM Ultraglutamine, 15 mM HEPES and 1% GIBCO antibiotic-antimycotic cocktail. DMEM/F12 \(Dulbecco´s Modified Eagle´s Medium, Ham´s F-12 1:1 mix with 15 mM Hepes and L-Glutamine) \(Lonza, Cat. N°.BE12-719F) supplemented with 10% Fetal Bovine Serum \(FBS) and 1% GIBCO antibiotic-antimycotic cocktail.

Equipment

EQUIPMENT SETUP Cell culture incubator with both, temperature and gas composition controls, was set at 37°C and 5% CO_2, \(New Brunswick and Eppendorf Company, Cat. N°. 00170S-230-1000) Biosafety cabinet suitable for cell culture and equipped with UV light for decontamination \(Polaris, Cat. N°. 11339) Vacuum pump ROCKER 300 \(Rocker, Cat. N°. 167300-22) Thermostatic bath Model IDL-AG12 \(Labolan, Cat. N°. 506012) Inverted contrasting microscope for living cell applications Leica DM IL \(Leica) Centrifuge 5810 R, bench top centrifuge, without rotor, refrigerated \(Eppendorf, Cat. N°. 5811 000.010) Fixed-angle rotor F-34-6-38 for Centrifuges 5804/5804 R and Centrifuges 5810/5810 R \(Eppendorf, Cat. N°. 5804727.002). Swing-bucket rotor A-4-62 for Centrifuges 5810/5810 R \(Eppendorf , Cat. N°. 5810 709.008). Confocal microscopy \(Leica TCS AOBS SP2)-40x oil immersion objective \(NA 1.25-0.75) was used and images were acquired using a 496 nm argon/krypton ion laser. The acridine orange signals were detected at 506-590 nm. Z-series profiles of an average of 20 optical sections were collected at intervals of 1 µm with a line average of 2 to reduce noise.

Procedure

PROCEDURE **Culture of cells-TIMING 30 min** 1│ Harvest cells growing in monolayer in T-75 ml cell culture flasks. Add 5.0 ml of trypsin-EDTA to each plate and incubate at room temperature for 4 min. Add

5.0 ml of RPMI 1640 with 10% FBS and 25 mM HEPES cell culture medium and transfer cell suspension into a 15 ml conical tube. 2│ Pellet suspended cells in 15 ml conical tubes using a centrifuge for 5 min at 500g. Re-suspend each cell pellet in 2-3 ml of media and perform cell counts. 3│ Place a coverslip inside culture plates \(6 well-plate) with sterilized tweezers. CRITICAL STEP. It is very important that all coverslips used for growing cells must be carefully handled to avoid contamination. Caution should also be taken when place the growing side \(usually only one side is treated for cell attachment) of the coverslip correctly. 4│ Complete media is then added to culture plates \(2 ml) without dropping media over the coverslips to ensure that cells keep attached to the surface. 5│ Cells are seeded at the appropriate final density \(50,000 cells/ml but may vary depending on cell type/size). After adding cells, plates are slowly shaken to homogenize cell distribution. **? TROUBLESHOOTING** 6│ Cells were left growing for 48 hours inside cell incubator. **Staining with fluorescence molecule- TIMING 20 minutes** 7│ Add 2 ml of fresh RPMI medium with 0.2% FBS in new 6 well plates. Three of the wells are used for washing \(PBS-glucose) while the other can be used employed for staining. 8│ Add 20 µl of AO solution to RPMI medium of three plates and shake to homogenize. 9│ Using sterilized tweezers place each one of the coverslips containing cells in one of the wells with washing solution for 1 minute and then take coverslips into wells for staining during 10 minutes at RT. 10│ After this time pipet 100 µl of RPMI medium with 0.2% FBS on a clean slide and then place coverslips with cells over medium upside-down. Then mounting medium is used as adhesive, using it only in the external limits of coverslip to avoid auto-fluorescence. 11│ Immediately after mounting place slide with one drop of immersion oil under the confocal microscopy. **? TROUBLESHOOTING** POINT WITHOUT PAUSE- It is strongly required to start collecting images from confocal microscope immediately after mounting. Therefore we do recommend mounting slides in the same room where confocal analysis is done. **Capturing images with confocal microscopy** 12│ Run the software with the setting up indicated above \(see equipment setup). 13│Search area of interest to include as many individual cells as possible. 14│ Indicate the coordinates to make the slices for analyzing. 15│ Establish 1 µm depth slices and store images for further processing 16│ To ensure reproducibility, repeat the process described in steps 12-14 until a number over 100 cells is reached. **Image data preparation** 17│ Run the 'Imaris' program for processing images. 18│ In the menu 'File', select 'Open' and click in the first section image, so the software will open all section images taken. 19│ In the new window that pops-up, select 'volume', 'surface' and then click on the \`blend' button . In the keyboard press 'Ctrl+D' and a new window pops-up. In this new window select "auto blend" and then close this window. 20│ Now, select 'surface' and in the flag of 'create' press two times blue bottom of play. 21│ In this step it is possible to modify the absolute intensity but we rather recommend to click the 'automatic mode' so we minimize manipulation of images. 22│ Click again on the 'play' button and a new window will pops-up filled with several lines corresponding to the different cell volumes. You must move the threshold line up in order to eliminate those objects that correspond to incomplete cells with too low volumes or "cut-off" cells on both sides of image selected. Then click again on the blue button to finish these steps. 23│ Select tab labeled as 'pencil' and click on 'select' for selecting all those incomplete cells that were impossible to eliminate in the previous step. For eliminating those cells just select them and then 'delete'. **? TROUBLESHOOTING** 24│ When working with specific cell types, many of them grow either in clusters or they form groups in which many of the cells remain bound one to each other. When

this happens, it is necessary to cut between two or more cells. However it is quite easy to identify the binding area because usually it appears as a "valley" just between adjacent cells thus indicating the joining point. Consequently user has to choose these points and make the appropriate cuttings for obtaining accurate measurements. **? TROUBLESHOOTING** 25│ Once user has completed this part of the software image processing of all cells, all data have been acquired by computer. To obtain data, select tab labeled as 'statistic' and then 'detailed' and choose the desired parameter such as 'volume', 'surface' or 'sphericity'. A table containing data will then appear. Just click on any of the data displayed and then the corresponding cell image will appear, so user can easily correlate any of the data with the cellular source. 26│ Data can be easily exported to an excel datasheet by selecting in the same tab the 'save' command. A new window will then appear with the corresponding data. 27│**OPTIONAL STEP**; It is recommended to save a copy of image using the 'snapshot' command. This will save pictures as a 'tiff' file which can be further used under any other program for treating images \(i.e. Photoshop, Paint Shop, etc…). This would allow users to compare and correlate data with cells using other programs with a more intuitive interface.

Timing

Culture of cells-TIMING 30 min-48h \(Steps 1-6) Staining with fluorescence molecule-TIMING 20 min \(Steps 7-11) Capturing images with confocal microscopy -TIMING 15 min \(Steps 12-16). Without repetition Image data preparation -TIMING 20 min \(Steps 17-27. Without repetition.

Troubleshooting

TROUBLESHOOTING PROBLEM: Step 5 and 24-Density of cultured cells. Too many/few cells appear at final magnification. SOLUTION: The density of cells is crucial at this point since the main objective is the real morphology of cells and therefore the treatment of images coming from real cells. To do so it is highly recommended to seed a few cells initially so image handling is easier when cell density is much below confluence. If cell density is near 90-100% it becomes difficult to isolate cells without cutting joints between cells. Otherwise it would be time consuming and not easy to obtain clean images and reproducibility is also lower. PROBLEM: Step 11-Speed too low while manipulating coverslips and confocal microscopy. SOLUTION: At this step when confocal microscopy is ready if operator is quick enough it is then feasible to scan more than five fields in the same coverslip in a minimum time. Otherwise, if all these steps are performed too slowly, only 2-3 fields can be analyzed and more coverslip are required to scan to obtain optimal results. The stopping point is indicated by the presence of bubbles or any other visual artifact, as this is an indicator that cells are not kept under normal conditions. PROBLEM: Step 14 and 23-Height of cells out of range. SOLUTION: When choosing low and high limits in z-axis for cell screening \(step 14), some of the cells are placed too up or down and consequently the whole cell is not contained in the gap. In these cases cells should not be considered for quantifying and operator must eliminate them manually at step 23. PROBLEM: Step 17 to 27-Treatment of images1. SOLUTION: Although it might be deduced that these steps depend highly on operator, following a few

indications ensures that the whole process becomes more reproducible. By comparing two independent observers here we demonstrate that differences obtained in cells parameters after the whole process is completed are below 1%. To this aim it is very important that all steps are set in automatic mode. If operator changes intensity manually in the software, care should be taken so intensity must be changed accordingly for all the measurements, otherwise process is subjective. On the contrary, setting mode to 'automatic', measurements are performed equally in all the cells. All these steps are further explained in a tutorial video shown as supplementary material video 1.

Anticipated Results

ANTICIPATED RESULTS For several researchers it may be very important to know exactly the intracellular concentrations of exogenous and endogenous compounds, proteins, DNA, RNA or any others molecules of interest. Currently there are several analytical applications for estimating the presence and the amount of virtually any molecule inside cells[11] . On the contrary, scientists do not have simple tools to estimate cell volume. To fill this gap, the primordial objective in this work was to create an easy, reproducible and accurate method to calculate volume of cells. Thus, in combination with HPLC or any other analytical method, the protocol shown here will allow estimation of intracellular concentration of molecules. Results with data of volumes from different cell types using the protocol reported here are shown in table 1. As it can be seen in table 1, it is remarkable the differences in cell volumes measured in different cell types. In some cases, differences of volume can be observed at microscopy level because cell diameter is the major source of variation. However, in other cell types diameter do not vary significantly and differences observed are rather due to cell height so our protocol would be especially useful for the latter. Protocol is easy to perform with most cells. Other cell types like murine macrophages RAW 264.7 cells offer particular difficulties. RAW 264.7 macrophages are easily activated by LPS or other factors including high cell density. When cells are activated they change dramatically in morphology which can be easily followed up under microscope. This particular feature was also identified using our method as it can be seen in table 2. In this case we observed that when RAW 264.7 cells were cultured at a density near 70% a high error in cell volume was noticed. This was due to both morphology and volume changes. When those "activated" cells are identified and processed apart \(table 2, see RAWc), the volume of the rest cells \(RAW[b]) are virtually the same that in other tests \(see table 1 for comparing). Furthermore with this data we can assure that morphology changes can be identified and even quantified. Furthermore, although we have mainly emphasized the use of this method for cell volume determination, it is possible to quantify other geometric parameters such as surface area or sphericity. In the latter case, sphericity, it can be used for quantifying morphological changes in which exogenous stimuli trigger changes in morphology or for quantifying differences of morphology between cell types. Figure 3 shows an example of how the protocol may illustrate and quantify the differences in cell morphology by comparing the 3D image reconstruction. For this purpose, we have provided videos 2, 3, 4 and 5 as supplementary material, where differences in cell morphology between cell types can be observed. Furthermore video 6 shows a 360° rotation of PC3 cell around other cells. All data and supplementary material shown here demonstrates that the protocol shown here is a simple, fast and

accurate tool for measuring different cell parameters in several cell types using a common CLSM which is accessible to most laboratories. More over in combination with other techniques available for many researchers, intracellular concentration of substances or any other comparative analysis of morphological changes can be easily performed. Currently many therapeutic approaches of how new drugs can be useful either in cancer, neurodegeneration or many other pathologies usually require some knowledge about how these substances enter into cells/tissues and what is the real concentration reached in those tagets. In fact the intracellular concentration truly discriminates between successful new drugs and the rest. However most researchers do not approach the possibility of establishing intracellular concentration of molecules because of the intrinsic difficulties in obtaining cell volume measurements. Therefore, the method proposed here may fill this gap and offers an easy and inexpensive way to overcome those difficulties.

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Acknowledgements
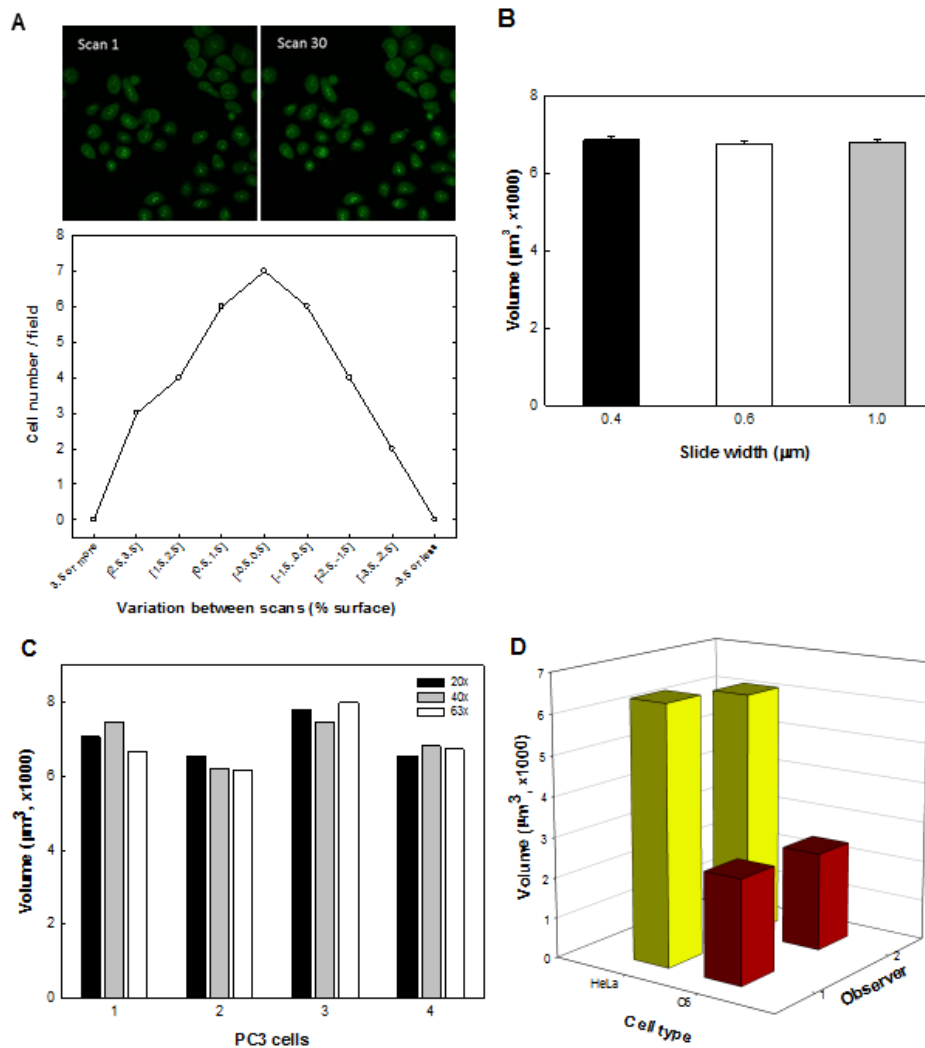
Figures

Figure 1

Test for potential error sources in the CLSM with 3D image processing method for determining geometric parameters in living cells A, Comparative fluorescence in PC3 cells stained with AO between scans 1 and 30 (top micrographs, x400) and the resulting graph comparing number of cells per field showing changes in surface area in each slide between -3.5% and 3.5% of total. B, PC-3 cell volume data from 30 PC3 cells using different width of slides (0.4-1.0 µm). C, Analysis of PC3 cells (n=4) when different objectives are used. D, Cell volume data from two cell lines, HeLa and C6 glioma cells, when the whole protocol is performed independently by two observers.
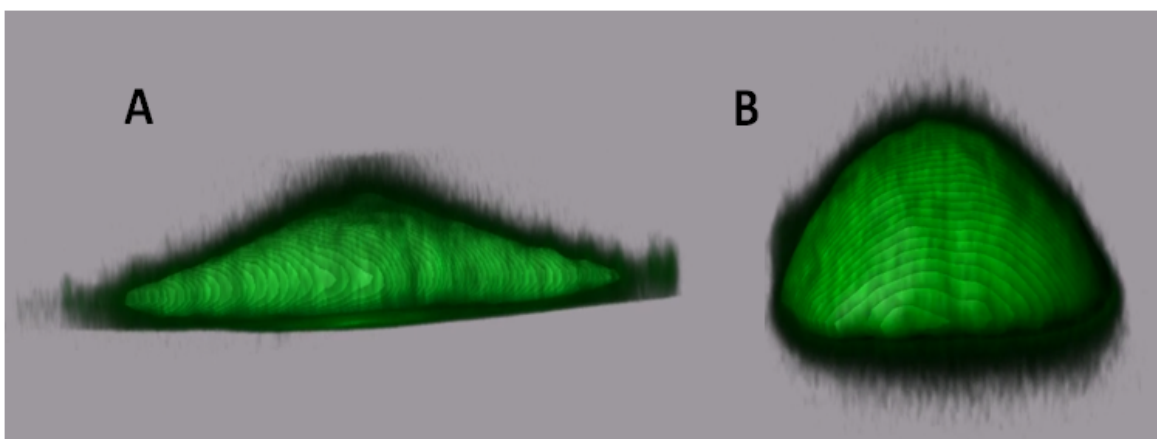
Figure 2

Figure 3 3-D reconstruction images from PNT1A (A) and PC-3 (B) cells using the protocol reported here.

Cell type	Volume (μm^3)	Error (% error)	N° of cells
PC3	6861.02	150.34 (2.19)	157
LNCaP	4675.04	139.62 (2.99)	111
DU145	4649.18	195.46 (4.20)	127
PNT1A	4219.10	130.09 (3.08)	159
C6	2530.11	84.54 (3.34)	221
B16F10	6441.92	199.36 (3.09)	163
RAW 264.7	2300.65	50.08 (2.18)	177
NIH	2909.92	94.85 (3.26)	125
CHO	2713.58	63.84 (2.35)	125
HeLa	5079.80	226.04 (4.45)	151
MCF-7	4858.15	114.61 (2.36)	148

Figure 3

Table 1 Cell volume Cell volume (µm^3^) as estimated by the protocol reported here, using several common cell lines. Mean volume, SEM and the number of cells used for each determination are shown.

| Cell type	Volume (μm^3)	Error (% error)	Nº of cells
RAW[a]	2938.71	321.29 (10.93)	75
RAW[b]	2276.28	98.84 (4.34)	69
RAW[c]	10114.95	2318.75 (22.92)	6

[a], whole RAW 264.7 cell population.
[b], subpopulation of not-activated RAW 264.7 cells.
[c], subpopulation of activated RAW 264.7 cells only.

Figure 4

Table 2 Differences observed in cell volume data from the same cell line. a, whole RAW 264.7 cell population. b, subpopulation of not-activated RAW 264.7 cells. c, subpopulation of activated RAW 264.7 cells only
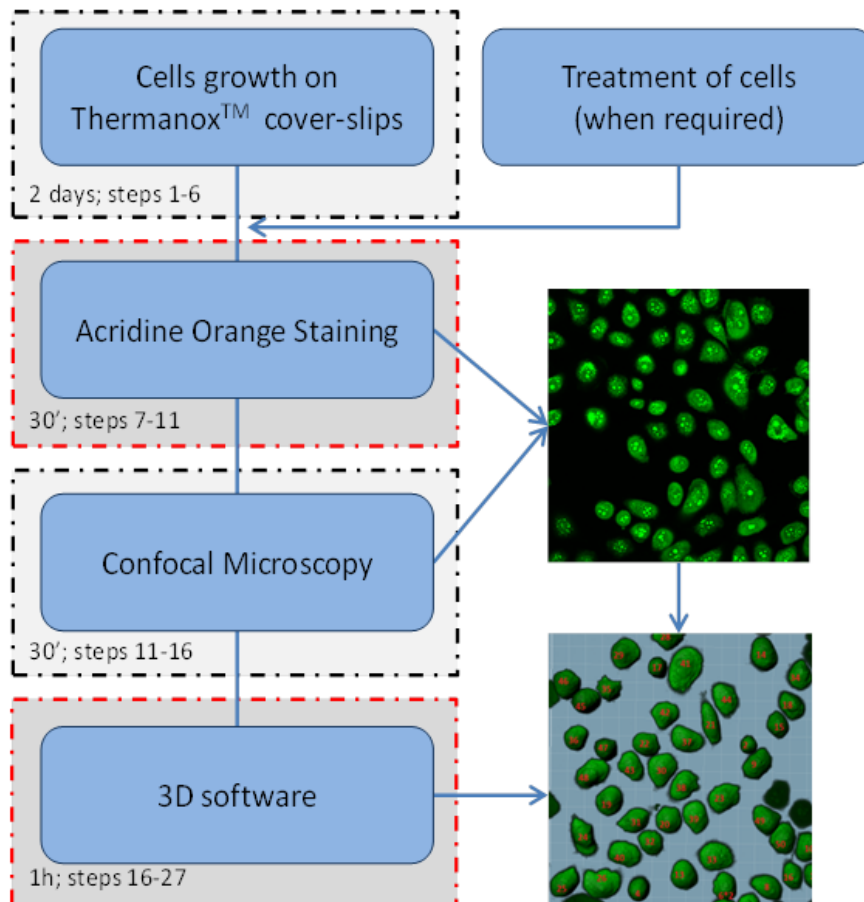
Figure 5

Figure 2 Outline of method Diagram showing the simple workflow process for the method described here, from cell seeding to data processing and the corresponding timing.

Supplementary Files

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