Research Article

High-resolution 3-D imaging of living cells in suspension using confocal axial tomography

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Conventional flow cytometry (FC) methods report optical signals integrated from individual cells at throughput rates as high as thousands of cells per second. This is further combined with the powerful utility to subsequently sort and/or recover the cells of interest. However, these methods cannot extract spatial information. This limitation has prompted efforts by some commercial manufacturers to produce state-of-the-art commercial flow cytometry systems allowing fluorescence images to be recorded by an imaging detector. Nonetheless, there remains an immediate and growing need for technologies facilitating spatial analysis of fluorescent signals from cells maintained in flow suspension. Here, we report a novel methodological approach to this problem that combines micro-fluidic flow, and microelectrode dielectric-field control to manipulate, immobilize and image individual cells in suspension. The method also offers unique possibilities for imaging studies on cells in suspension. In particular, we report the system’s immediate utility for confocal “axial tomography” using micro-rotation imaging and show that it greatly enhances 3-D optical resolution compared with conventional light reconstruction (deconvolution) image data treatment. That the method we present here is relatively rapid and lends itself to full automation suggests its eventual utility for 3-D imaging cytometry.

Keywords: 3-D reconstruction · Deconvolution · Dielectric field caging · Flow cytometry · Micro-rotation

1 Introduction

Conventional flow cytometry (FC) is ideal for simultaneous analysis of multiple fluorescence signals in single living cells in suspension. FC techniques are especially attractive because of their potential for high-throughput operation wherein averaged fluorescence can be quantified from thousands of (particles) cells per minute or even in some cases per second. Further, this high-throughput mode of operation is compatible with cell sorting and viable cell recovery methods [1, 2]. However, a major constraint of FC is that signal readout is limited to fluorescence intensities averaged from the entire cell and integrated to a single value, whereby potentially important spatial information is lost. In the worse case scenario a phenotype characterized by reorganization of compartmentalized signals, with no alteration in fluorescent signal intensity, will not be efficiently or readily detected by FC. This limitation has been partly overcome by the recent introduction [3, 4] of imaging FC (IFC) methods whereby high-speed digital images of single cells are recorded simultaneously across multiple fluorescent channels (up to six) without too much compromise on throughput rates (i.e., up to 10 000 cells/min). This is a major advance because
it allows information on the spatial subcellular distribution of fluorescence to be recorded and interpreted in addition to simple fluorescence intensity read-out. In many ways IFC may therefore be considered a high-content and high-throughput imaging method allowing image-based detection of multiplexed fluorescent signals from many cells automatically at high speed.

Despite advances in the current state of the art for IFC, the ability to extract 3-D spatial information from cells in flow suspension (ideally in a FC context) remains an unsolved and difficult problem. 3-D imaging of microscopic subcellular spatial details requires somewhat specialized equipment and methods. Specifically, a microscope equipped with a high magnification objective (and high numerical aperture), precision motorization for high-speed nanometer z-axis displacement, and a high-sensitivity high-speed digital camera are required. In turn, these electromechanical- and optical-hardware elements must be coordinately piloted in a precise protocol whereby a so-called axial “through-stack” image series (or “z-stack”) is recorded from target samples immobilized upon a thin, optical quality, glass slide. The axial through-stack data may then be processed to extrapolate 3-D information [5]. Analysis of 3-D light signals often uses mathematical light reconstruction (also known as “deconvolution”) techniques that enhance signal-to-noise ratio and image feature contrast by helping to remap so-called “out-of-focus” light contamination in the through-stack using prior knowledge of the microscope’s optical characteristics [6–9]. This type of multiple-dimension (multi-D) fluorescence imaging microscopy is the standard approach for microscopic 3-D cellular imaging, and is an essential tool for cellular biology, and infection [10, 11] inasmuch as the resolution of 3-D information impacts importantly on our understanding of the underlying biological structure and functions we observe [12–14].

It is important to appreciate that conventional through-stack multi-D imaging has some significant limitations. Among the main practical constraints selected target cells must be attached (immobilized) on an optically transparent substrate. Many cell types are functionally nonadherent, and others are certainly in a functionally altered state upon adherent attachment [15–17]. It is noteworthy that among the 3600 cell lines harbored in the depositories of the American Type Culture Collection, there are at least 1400 cells described as displaying distinct growth properties “in suspension”, that is to say that they present phenotypes related to nonadherence. Evidently, these cells maintained in suspension comprise a stockpile of cells that have yet to benefit precise 3-D image analysis. Indeed, the dependency of 3-D imaging techniques on sample immobilization has resulted in there being relatively few published studies using high-resolution 3-D interrogation of cells maintained in suspension. As an alternative to cell immobilization by adherent attachment to an optical substrate, dielectric field cage (DFC) technology has been demonstrated as a useful tool for manipulation of living cells in suspension. The principle of DFC is that charge-bearing particles (e.g., individual cells) suspended in a low ionic medium can be perfused between micro-electrodes carrying a low-frequency (kHz range) alternating-current (AC) drive wherein charge-bearing particles experience a dielectric charge shift (dielectrophoresis) that, in this context, generates a dynamic physical inertia. Inside a 3-D (octode) cage, the phase of AC at opposing electrodes may be modulated in a manner allowing an individual cell to be gently manipulated in 3-D space and micro-rotated in micrometer dimensions. DFC technology is attractive for 3-D microscope imaging because it offers a facile means to immobilize target cells in suspension [18–21]. Further, combined with micro-fluidic flow control, DFC cell chambers individual cells can be rapidly introduced into the octode cage traps, imaged, and then exchanged for a new cell in a matter of seconds; in addition, we have recently shown that downstream automated cell recovery is possible [22]. Here, we describe for the first time a complete method and validation for 3-D axial tomography using dielectric micro-rotation imaging of living cells in flow suspension.

2 Materials and methods

2.1 Cell line preparation

Human tumor cell lines SW13 (adrenocortical carcinoma) were maintained in DMEM medium (Gibco) supplemented with serum and antibiotics. SW13/20 cell line (a kind gift from Christopher Hutchison, [23]) were chosen because they display stable, high expression levels of a nuclear lamin-A fused with green fluorescent protein (GFP), resulting in strong fluorescence patterns tightly localized to the nuclear envelope and easily detected in most cells. Before observation adherent cells in culture were washed in phosphate-buffered saline (PBS), then trypsinized for 5 min at 37°C and subsequently resuspended in Cytoscan™ buffer II (Evotec Technologies/Perkin Elmer) at a final concentration of 10^5–10^6 cells/mL. The electrical conductivity of the iso-osmolar Cytoscan buffer was 0.3 S/m.
2.2 Fluorescent bead preparation

Green fluorescent beads of 15 μm diameter (FocalCheck, Molecular Probes/Invitrogen) were either attached to a coverslip or trapped in suspension. For imaging beads attached to a coverslip, 0.5 μL of the stock solution was deposited upon the cover glass at the bottom of a customized culture dish (MatTek Corp.) and left for several minutes to dry, before adding 500 μL Cytocon™ buffer II. For imaging beads in suspension, 50 μL of the bead stock solution was diluted 1:4 using Cytocon™ buffer I.

2.3 Acquisition of stepwise micro-rotation time-lapse image series

The method for handling and trapping living cells in DFC3 chips has been described previously (for example, [22, 24, 25]). Briefly, suspended cells were allowed to flow into a microfluidic chamber (DFC-3 chip; Evotec Technologies, a Perkin Elmer company) using a "T" injector and a 10-µL syringe (Hamilton, Switzerland). The electrodes of the octode cage are separated by 40 µm from tip to tip and are 40 µm in height. Inside the cage isolated cells were trapped in suspension, 50 μL of the bead stock solution was diluted 1:4 using Cytocon™ buffer I.

2.4 Image processing

Single through-stacks were deconvolved using Huygens Essential software (SVI). Theoretical resolution estimates for different optical set-ups used the freely available “Microscope Resolution Calculator v1“ (http://www.pfid.org/html/objcalc/?en). For estimating quantitative enhancement in data quality, we calculated what we termed “empirical contrast” (EC) as the averaged signal intensity in any given region-of-interest on the imaged object divided by the averaged background measured in a region of interest outside the imaged object. Where stated, correlation coefficients were calculated using Pearsons’ method in Image J (plugin: Image correlationJ [28]). Other image processing used Image J standard toolbox (Rasband, W. S., ImageJ, U.S. National Institutes of Health, Bethesda, MD, USA. http://rsb.info.nih.gov/ij/ 1997–2006). Graphs were processed using Excel software (Microsoft). Figure montages were created using Adobe Illustrator CS2.

3 Results

3.1 Conventional axial through-stack imaging of DFC trapped micro-objects in suspension

We sought to establish conditions wherein micro-objects (either fluorescent plastic beads, or cells) could be trapped in a sufficiently stable manner to allow conventional 3-D through-stack imaging. Our criteria used comparison of 3-D through-stack reconstruction from images collected in convention-
al mode (sample-substrate adherent) with through-stack series recorded from comparable objects suspended momentarily inside the dielectric field trapped in fluid suspension. We observed no qualitative differences between through-stack reconstructions from beads trapped in the DFC cage in suspension, and beads attached to the glass substrate (Fig. 1), and this held true for both oil and water immersion objectives (that provide different theoretical correction factors). We did not detect any gross misalignments among stack slices in the same series as evidenced by the symmetry and smoothness of 3-D reconstruction of data recorded from fluorescent plastic beads; and there were no differences detected in the extent of axial (z-axis) aberration apparent in each modality again independent from immersion medium, objective and numerical aperture (Figs. 1A–D'). From these results we concluded that the stability of micro-objects held in suspension inside DFC cages is wholly sufficient to support conventional through-stack acquisition.

3.2 Reproducible stepwise micro-rotation of individual living cells trapped in suspension

Modular forces exerted by control of phase between AC at microelectrodes in the trapping cage could be re-oriented geometrically to angles perpendicular to origin. Inasmuch as the octode trapping cage has orthogonal symmetry (Fig. 2A), this allowed us to easily set up a protocol that could rapidly (<10 ms) switch and thereby rotate individually trapped cells in a stepwise perpendicular manner. This allowed optical imaging to be performed from several points of view distributed around 360° (Figs. 2B and C). We next investigated the reproducibility of this stepwise micro-rotation protocol by measuring the pixel intensity correlation registered between the images recorded first preceding rotation, and then following four stepwise perpendicular shifts, each step executed every few tens of seconds (Fig. 3A schematic). As illustrated (see color merge Fig. 3C) the averaged correlation coefficient (0.908 ± 0.058; n=5) indicated a high-degree of pixel intensity (i.e., feature) match between images of the cell following 360° rotation through four stepwise maneuvers. For comparison, the correlation (0.976 ± 0.009; n=5) calculated from cells trapped statically in a single point of view orientation and from which four image stacks were recorded at comparable time intervals but without micro-rotation (Figs. 3B and E) was not different. Further, both these values differed significantly from the correlation (0.242 ±0.078; n=5) detected in images recorded from cells trapped in two perpen-

Figure 1. Through-stack imaging of 15-μm green-labeled beads. Through-stack fluorescence image series were recorded from a single bead (FocalCheck fluorescent microspheres, Molecular Probes) using a spinning disk confocal microscope (Andor Technologies, Revolution XD) equipped with an objective piezo-drive. Beads were imaged either immobilized by attachment to a cover-glass (A, B), or by DFC trapping in suspension (C, D). In both cases image acquisition used either a 63× oil 1.4NA (A, C) or a 63× water 1.2NA (B, D) objective. A single XY (lateral) optical slice selected from close to the center of each cell sampled is presented (A–D). In addition, using the respective through-stack series an orthogonal XZ slice view was generated, corresponding approximately to the physical center of each bead (A–D) along the Y axis (A'–D'). Red dotted line separation represents 15 μm (continuous red line is in the middle). A blue circle represents the real shape of the bead as it would be seen without axial aberration. Beads were suspended in medium with a refractive index of 1.34. In all cases, the z-step is 100 nm and the scale bar (white line in A) represents 5 μm.
dicular positions where the RGB merge revealed the expected mismatch (Fig. 3D). These results support the conclusion that DFC based micro-rotation facilitates reproducible 3-D re-orientation of living cells trapped in suspension.

3.3 Registration of multiple axial through-stacks

The stability of 3-D trapping in suspension, combined with the ability for rapid perpendicular re-orientation of living cells inside the trap led us to next examine a protocol for axial through-stack sampling from multiple (at least two) perpendicular oriented points of view. As shown in the schematic view (Fig. 4A), we reasoned that individual living cells trapped in suspension inside the octode cage could first be through-stack sampled in
any given position, re-oriented using a 90° field rotation command, and again through-stack sampled; and that the two perpendicular “z-stack” view points could be combined into a 3-D reconstruction. As expected, DFC trapping served well to re-orient individual cells. However, the precision of the micro-rotation step showed small, but not insignificant deviations from 90° due mainly to the dependence of the field control on the specific shape of each target cell. To address this problem (i.e., the uncertainty of the rotation angle, which could diverge as much as 20° from the target value of 90°), we used a mathematical algorithm to treat image data stack pairs, with the aim of allowing any arbitrary point of view (or slice) to be precisely (with sub-pixel resolution) registered. A representative example result from this type of image processing treatment illustrates its efficacy (cf. Fig. 4B with 4C). In this example, two stacks were recorded from different angles of view as illustrated in the schematic (Fig. 4A). The second stack (Fig. 4C) was recalcualted rapidly (a few hundred seconds on a standard desktop processor) to align rigid 3-D feature space (see Materials and methods) with the first stack (corresponding orthogonal views Fig. 4B). Conveniently this registration method also allowed us to extract the exact angle of rotation, which in this case was 105.42°, and under most normal conditions ranged between 70°–110°.

3.4 3-D light reconstruction of registered axial through-stacks

Having registered the image stacks and extracted the precise angle between them, we next used a second image-processing step for treatment of multiple stacks for simultaneous stack-fusion and deconvolution. A representative example result is shown where the first through-stack (from Fig. 4B) and the second through-stack (from Fig. 4C) were combined into a single deconvolved stack. The resulting “fusion-deconvolution stack” displayed a clear and distinct improvement in image quality over raw data (Figs. 4B, 4C cf. 5’). Inasmuch as the data treatment represents a deconvolution treatment, the result (Fig. 5A) is compared with a conventional deconvolution counterpart, from the original data (z-stack 1; Fig. 5B). Here, the optical aberration observed in axial views of the conventional deconvolution stack (vertical smearing of image, Figs. 5B’ and B”) is almost completely absent in the corresponding views of the fusion-deconvolution (Figs. 5A’ and A’). These improvements in
data quality were also apparent in 3-D reconstructions, which were greatly improved in the case of fusion-deconvolution (see supplementary Fig. S1) wherein both 3-D-volume and 3-D-surface rendering showed reduced axial aberration and improved fine feature detail.

We attempted to quantify the quality improvement resulting from fusion-deconvolution by comparing what we termed the EC (see Material and methods) calculated for different data processing regimes (Fig. 6). Fusion-deconvolution treatment routinely yielded a multiple-factor-fold (>1000 times) enhancement of image contrast (EC ratio compared with raw data, see Fig. 6B). Further, even compared with conventional deconvolution treatment per se we routinely observed >100 times increased EC ratio enhancement (Fig. 6B). Finally, the increased EC ratio was accompanied by a comparable improvement in fine-feature delineation, evident to examination by eye as a greatly enhanced image quality (Fig. 6A, see also supplementary data movie S2).

4 Discussion

4.1 3-D imaging cytometry

We have developed an approach facilitating conventional axial through-stack imaging applied to living cells in suspension. Further, we demonstrate that with appropriate mathematical methods multiple viewpoint through-stacks can be deconvolved to yield a remarkable 3-D image enhancement.
These results impact the current state of the art for 3-D imaging of live cells in two major ways: first, by providing what may be considered a new 3-D imaging modality for microscopy; and second, by giving a methodological basis to future development of automated 3-D imaging cytometry.

4.2 Micro-rotation imaging: 3-D reconstruction using confocal axial tomography

Conventional axial through-stack imaging combined with deconvolution processing is a powerful means to interrogate microscopic details in a 3-D volume such as a cell. Our method significantly enhances the power of this approach by allowing quantitative interpolation of data content extrapolated from two independent axial through-stack viewpoints recorded from the same target. The rationale of this approach has been previously considered in studies proposing an approach for so-called 3-D axial confocal tomography that used a fusion-deconvolution algorithm [29, 30]. However, in those studies the method for the acquisition process used cells fixed to a glass capillary that could be rotated only through a constrained geometry, and through-stack sampling was slow due to the need for image averaging and external mechanical movement [29]. The DFC technology presents a faster and more dextrous means for rotational cell manipulation and, moreover, does not require target cell adherence to glass.

We have recently reported that the original [29, 30] mathematical method for confocal axial tomography by through-stack fusion-deconvolution also works well on micro-rotation data without extensive adaptation [22], with the caveat that the angle of rotation between the two through-stacks was unknown, a fact that limited the potential accuracy of the reconstruction. In DFC micro-rotation the angle of rotation must be estimated or calculated empirically. In the current study we therefore capitalized on the use of an as-yet-unpublished and to our knowledge unique through-stack image registration algorithm that allows the angle of displacement between the two through-stacks in 3D space to be precisely calculated. Thus, a major improvement in the method presented in the current work comes from the through-stack registration preprocessing algorithm, with the ability to accurately measure angular rotation and thereby register the two-through-stacks recorded during stepwise micro-rotation, followed by reconstruction using our own customized version of the fusion-deconvolution treatment [29, 30]. As a result of the ability to precisely register the data and based upon empirical analysis, we estimated that fusion-deconvolution resulted in factor-fold increase in image contrast compared to raw data treated by conventional deconvolution, in a manner suggesting that the increased information in two stacks improved the probabilistic estimate underlying the 3-D light reconstruction process (deconvolution). This interpretation is consistent with the apparent increase in resolution detected by eye, and confirmed by Fourier analysis, where increased high-frequency information was observed (data not shown). The remarkable enhancement of EC ratio exerted by axial fusion-deconvolution suggests that photons are better reassigned by light reconstruction using dual-stack fusion-deconvolution, in a manner assuring an improved overall performance for the imaging system. Along these lines we have yet to examine the exciting possibility that this could provide a powerful means to enhance the photon statistics of the imaging system's overall performance to an extent whereby we could then reduce the acquisition times by at least a factor fold without compromising data image quality.

4.3 A method for 3-D imaging cytometry

The method we present here appears to provide a basis for 3-D imaging cytometry. Along these lines an especially attractive aspect of this approach is rapid (seconds) acquisition and reconstruction (hundreds of seconds). Albeit still far from the high-throughput of flow cytometry, we expect acquisition times to be rapidly reduced to less than 10 s/cell, and still further in the near future. In an automated configuration this would therefore provide for high-content analysis with adequate throughput. It is sobering to consider that the current state of the art for high-content analysis of cells in suspension provides 2-D IFC analysis at throughput rates around 5000–10 000 events/min (6–12 ms/event). Micro-fluidics combined with DFC are currently far from achieving this target. However, in lieu of improvements along these lines, our results already provide the first proof in principle for 3-D imaging of living cells in flux suspension, demonstrating at least a basis for technology development towards automated 3-D imaging cytometry technologies. Whether development of micro-fluidics DFC-based methods would eventually allow throughput comparable to FC/IFC is doubtful, inasmuch as these approaches are fundamentally different in a number of important aspects that distinguish their application. First, due to the high flow rates necessary to the high-throughput of FC/IFC technologies, living cells (especially primary cells, and cells made fragile due to infection or other pathological conditions) can become diffi-
cult to maintain in a viable state after cell sorting, especially when handling very small cell populations. Micro-fluidics combined with DFC is extremely gentle due to its low-throughput nature, and both semi-automated [22] and fully automated [31] cell recovery display high viability. Second, like most high-throughput high-content imaging methods IFC has to compromise optical resolution in favor of speed. For example, some of the best commercial IFC systems available offer optics with relatively low numerical aperture (e.g., NA=0.75). In effect this will strongly diminish the lateral and axial resolution of the imaging system by at least twofold (comparing for example a 63×, NA=1.2 water immersion to a 63×, NA=0.75 dry/air objective). By contrast, DFC-based micro-rotation imaging makes no compromise on resolution and the imaging optics, etc., are not at all restricted. Third, IFC data do not lend themselves to quantitative 3-D light-reconstruction, and instead use an extended depth of field optics to maintain focus throughout the sample volume. Micro-rotation by DFC combined with axial through-stacking preserves quantitative 3-D information on the target cell, with optimal resolution. Based upon these differences DFC-based micro-rotation looks set to fill a different niche to IFC technologies, where 3-D resolution and light sensitivity are of the essence. Such nominally ultra high-content applications would be those demanding high-resolution detail in favor of throughput. Further, it is noteworthy that the necessary technology where cell trapping, imaging and cell recovery are automated is already available commercially [31], but has yet to be fully developed for automated 3-D imaging. It is therefore not at all unreasonable to imagine in the near future automated micro-fluidic control and micro-electrode manipulation supporting 3-D imaging cytometry at low-throughput in the range of hundreds of cells per hour. The striking image quality gain afforded by micro-rotation-based axial tomography warrants its further development wherein automation should bring it to find routine application among exigent cell imaging laboratories.

In conclusion, axial confocal tomography by DFC micro-rotation provides us with a unique method for 3-D reconstruction of fluorescent signals in nonadherent microscopic objects. Notably, it actually enhances the quantitative precision of the 3-D reconstruction compared to conventional methods by enhancing 3D resolution. The method is made especially attractive in that both image acquisition and processing are relatively rapid. Combined with the fact that micro-fluidic handling, DFC cell trapping and micro-rotation lend themselves well to automation, these facts raise the promise that the method could eventually find utility in routine high-content cytological analysis. Thus, distinct from FC analysis that involves cell counting and averaged signal integration, our approach allows single cell cytological structure to be quantitatively interrogated in 3-D space.

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Conflict of interest statement: The registration algorithm and source code (authored by YY, AT and BC) is unreleased in observance to its international copyright protection under the auspices of CNRS (Centre Nationale de la Recherche Scientifique). The fusion-deconvolution algorithm and source code is unreleased, proprietary to Scientific Volume Imaging (The Netherlands). Finally, a joint patent [26] exists between the Institut Pasteur (Paris) and Evotec Technologies (Hamburg, a Perkin Elmer subsidiary).

5 References


