Cytometry PART A
ISAC Journal of Quantitative Cell Science

DEPArray™ System: An Automatic Image-Based Sorter for Isolation of Pure Circulating Tumor Cells

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Received 11 October 2018; Revised 2 November 2018; Accepted 5 November 2018

Grant sponsor: Electronic Components and Systems for European Leadership (ECSEL), Grant number: 661796

Additional Supporting Information may be found in the online version of this article.

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Published online in Wiley Online Library (wileyonlinelibrary.com)

DOI: 10.1002/cyto.a.23687

Abstract

Circulating tumor cells (CTCs) are rare cells shed into the bloodstream by invasive tumors and their analysis offers a promising noninvasive tool to predict and monitor therapeutic responses. CTCs can be isolated from patient blood and their characterization at single-cell level can inform on the genomic landscape of a tumor. All CTC enrichment methods bear a burden of contaminating normal cells, which mandate a further step of purification to enable reliable downstream genetic analysis. Here, we describe the DEPArray™ technology, a microchip-based digital sorter, which combines precise microfluidic and microelectronic enabling precise, image-based isolation of single CTCs, which can then be analyzed by Next Generation Sequencing (NGS) methods. © 2018 The Authors. Cytometry Part A published by Wiley Periodicals, Inc. on behalf of International Society for Advancement of Cytometry.

Key terms

circulating tumor cells; cancer cells; image-based sorting; pure cells; single cells; liquid biopsy; rare cells

CIRCULATING tumor cells (CTCs) are rare cells that are released and disseminated into the blood stream and lymphatic system by advanced cancers (1,2). CTCs can be isolated starting from whole blood, and liquid biopsy represents a simple and non-invasive alternative to conventional single-site biopsy. The multisite origin of CTCs and their molecular diversity can provide a full picture of cancer heterogeneity, describing the whole tumor genomic landscape (3–5).

Over the past decade, CTCs have become an attractive area of cancer research due to their potential clinical use for genome-driven targeted therapy selection and monitoring of disease progression (6). These principles, associated to the possibility to follow the course of disease by sequential sampling, represent fundamental requirements for the development of personalized treatment strategies.

Because CTCs are rare and heterogeneous, one of the key challenge for their clinical adoption is the development of standardized and reproducible technologies able to isolate and characterize these tumor cells (2,6,7). Several technologies to enrich CTCs from normal hematopoietic cells have been developed in the past years (1,8). These technologies rely on different working principles, mainly physical and biological properties of CTCs, including size, density, cellular charge, and specific expression of cellular markers. However, all CTCs enrichment platforms need a further step to isolate pure, single CTCs for molecular analysis (1,2) and thus, these methods need to be coupled to single cell sorting technologies. This is particularly relevant as the development of single-cell sequencing technologies has been recognized to be crucial for the study of tumor heterogeneity in the context of liquid biopsy (9) and contaminating DNA/RNA from leukocytes represent an important obstacle for the precise molecular characterization of CTCs.

In the last decade, our group have developed an image-based cell-sorting technology, named DEPArray™ (Menarini Silicon Biosystems, S.p.A., Italy), which

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synergistically combines microelectronics and microfluidics in an highly automated platform, enabling a simple and reliable way of isolating pure, single, viable rare cells from an heterogeneous sample, with unprecedent purity for molecular analysis (10–12).

Here, we describe the working principle of DEPArray™, the key steps of single-CTC purification and the potential role of this technology in the field of cell-based liquid biopsy for precision medicine.

Working Principle of DEPArray™ Technology

The DEPArray™ NxT System, the latest development of the technology, is composed of three elements: a benchtop instrument, a disposable microfluidic cartridge, and a proprietary software—the CellBrowser™ (Fig. 1A). The core of the technology is the microsystem cartridge, which is a single-use device integrating a microelectronic silicon chip, microfluidic chambers and valves. The working principle of the DEPArray™ is the Dielectrophoresis (DEP), an electrokinetic principle based on the ability of a nonuniform electric field to exert forces on neutral, polarizable particles, such as cells, which are suspended in a liquid. The silicon substrate in the cartridge integrates an array of over 300,000 micro-electrodes, each electrode can be programmed and energized with Alternating Current in-phase or counter-phase voltages with respect to the glass lid, which is conductive and transparent. By applying an appropriate pattern of phases, the array can generate up to 30,000 "DEP cages" in the Main Chamber, each one able to capture a cell in stable levitation, avoiding contacts between the cells and surfaces during the sorting process (Fig. 1B). DEP cages are able to trap and move cells

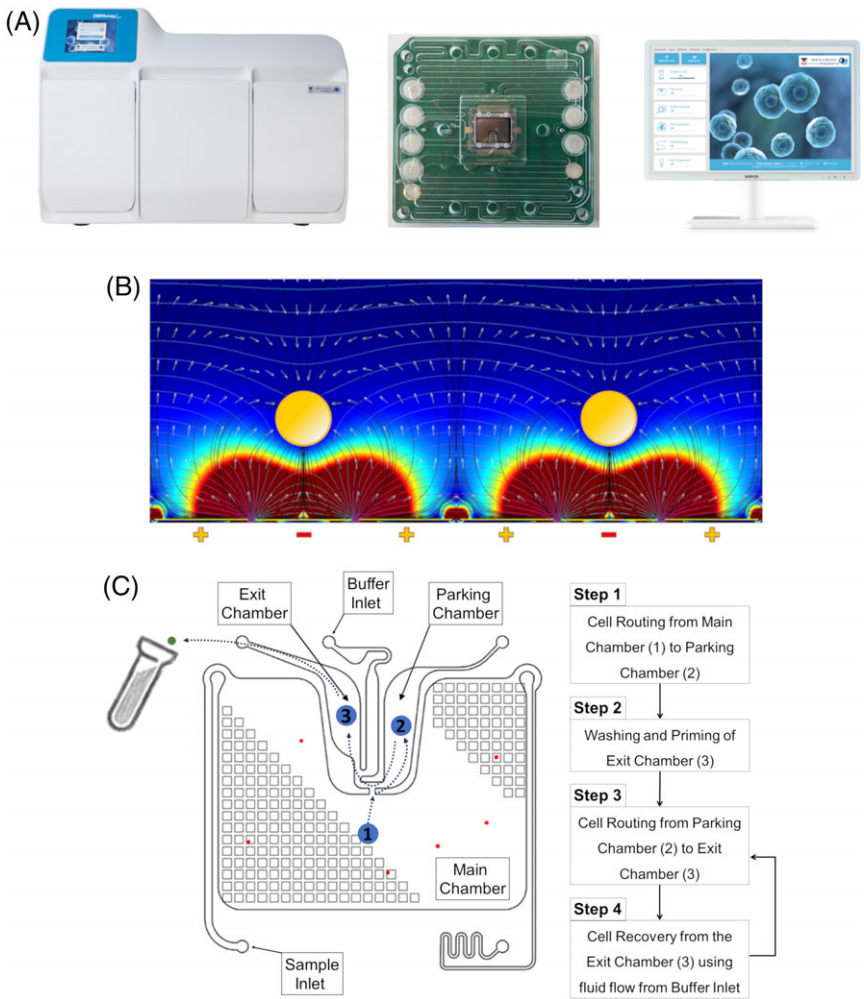

Figure 1. Main components of DEPArray™ NxT system (**A**). The benchtop instrument (left), the cartridge (middle) that combines microfluidics and microelectronics, and the CellBrowser™ software (right) to elaborate fluorescence and bright field images for automatic or operator-assisted cell selection. Schematic representation of dielectrophoresis showing the nonuniform electric field generated in the silicon chip that induces a polarization of cells, which are trapped in stable levitation (**B**). Schematic representation of DEPArray™ chip (**C**). After the phases of sample and buffer loading, the cells are randomly distributed into the Main chamber (1). Then, the selected cells are simultaneously moved toward the parking chamber (2). After a washing and priming phase of exit chamber (3), the cells are moved from the parking chamber (2) to exit chamber (3) for the cell recovery (4).

of different type and size ranging from small sperm cells to large epithelial cells (11,13,14). This electronic structure is integrated within an innovative microfluidic architecture that includes three micro-chambers in fluidic connection (Fig. 1C). The Main Chamber, where the sample is loaded, the Parking Chamber, where the target cells are collected before the recovery and the Recovery Chamber. The Main Chamber has a volume of 9.26 μl, while the volume pre-loaded in the fluidic manifold of the cartridge is 12 μl, resulting in a theoretical loading yield of 77%. The area of the Main Chamber comprises 30,000 cages, and assuming a random Poisson distribution this would imply that when the sample is unlimited, the optimal loading to obtain the maximum number of cells in a cage by itself would be equal to 30,000 cells (Average Cells Per Cage = 1), corresponding to 11,037 cages with single-cell. In practice, due to pseudo-random distribution and to take into account also other nonidealities of the sample such as clusters, we observed that best results are typically obtained with slightly lower cell numbers, such as 20,000, which according to Poisson distribution corresponds to 10,269 cells (51.34% with respect to the 20,000) in single-cell cages. In practice, carry over from blood enrichment with CellSearch® is typically lower, the entire sample is loaded and the predicted percentage of cells on a cage by themselves increases to 84.61% of 5,000 cells present in the Main Chamber, and goes up to 98.8% of 1,000 cells.

On the lower end of the range there is no actual limit to the minimum number of cells, even a single cell present in the Main Chamber may be detected and sorted. However, it should be noted that, according to the 77% Loading Yield, and modeling with a binomial distribution having probability of success P equal to the loading Yield, at least two cells should be present in the sample injected in the cartridge to have >90% confidence (actual probability 94.8%) that at least one is present in the Main Chamber. The study of minimum number of cells is of particular interest for assessing how many target cells given as input into the cartridge can be expected in the Main Chamber. For example, as reported in the corresponding statistical model of Supporting Information Table I, with a confidence level > 90% at least three cells would be redetected in the Main out of five cell present at input, at least five out of eight and at least six out of ten, respectively.

Detection and Isolation of Pure and Single Cells

On loading of the labeled single cell suspension in the cartridge, cells are randomly distributed into the Main Chamber and get trapped in stable levitation in the nearest DEP cage (Fig. 2A). In the DEPArray™ NxT, the illumination is provided by a stabilized LED lamp, which combines high efficiency excitation and emission filters, providing the possibility to get images in up to five fluorescent channels with negligible spill-over among the different channels (Fig. 2B).

The fluorescent microscope integrated in the DEPArray™ instrument allows acquiring high-resolution images (0.363 μm per pixel) for each individual cell in the sample thus enabling a high-definition analysis of in-cage events, detecting the specific expression of CTCs or no-CTCs markers. Bright field filter visualization coupled to fluorescence signals allows an accurate selection based on the combination of physical and biological parameters, including cell size, shape, circularity, and fluorescence intensity (Fig. 2D).

Beyond fluorescence quantification, bright field images are essential for the accuracy of the selection, when dealing with rare-cells such as CTCs enriched from blood. Co-localization of fluorescent signals across different channels, direct evaluation of size, roundness, and other morphological parameters, as well as the pattern of fluorescence are all key elements to distinguish target cells from spurious events (Fig. 2D). The proprietary CellBrowser™ software enables an automatic or operator-assisted identification of the desired cells through the elaboration of high-resolution images, minimizing the possibility to select inappropriate events, such as debris and doublets.

CTCs are identified by the expression of specific tumor markers, such as cytokeratin (CKs), avoiding the contamination of white blood cells (WBCs) that express CD45. The different cell populations are selected by using a manual or semi-automatic gating. Typically, CTCs are selected as $CK^{positive}/CD45^{negative}$ cells, while WBCs are selected among $CK^{negative}/CD45^{positive}$ cells (Fig. 2C), but other user-defined staining and identification strategies can be easily adopted.

Once identified, each target cell can be isolated from the bulk population, automatically, in the following way: the instrument moves the selected DEP cages (containing the target cells) by changing the electric field pattern step by step, deterministically, concurrently, and independently along trajectories calculated by the software, moving each selected cell from the original location into the Parking chamber. In the current version of the DEPArray™ cartridge, the Parking chamber can accommodate more than 1,000 "parking slots," and multiple groups of cells of different type can be parked simultaneously before recovery.

Afterwards, cells can be displaced, as single-cells or in pools of up to 507 cells. Typically, the process takes about 16 min for system calibration and Main Chamber loading, 20 min for image acquisition and analysis, 10 min for cell selection, 12 min for cell routing from Main Chamber to Parking Chamber, and 6 min for the priming and washing of the exit channel, resulting in a total time of 64 min (except for cell selection, all the steps are executed automatically by the system). The subsequent recoveries take 107 s for a single cell or 192 s for a pool of 20 cells.

At the end of the process, the target cells can be eluted from the device directly into various types of supports, through an accurate microfluidic control, by flowing clean buffer loaded in the cartridge prior to use. The recovery procedure can be repeated to obtain from the same sample multiple separate recoveries of individual target cells (up to 96) and/or groups of cells.

Accuracy of DEPArray™

Unlike other traditional bulk sorting, DEPArray™ technology isolates single and pure cell populations. For instance, CTCs can be purified individually, which is fundamental to

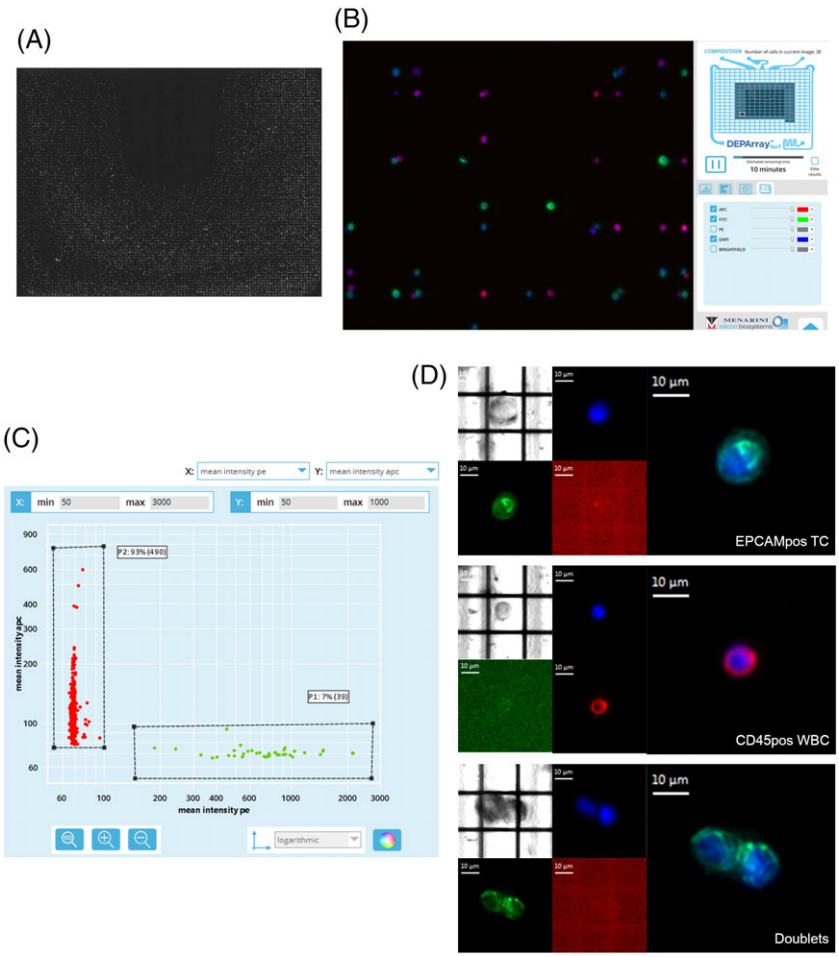

Figure 2. CellBrowser™ software images showing DAPI-positive cells randomly disseminated into Main chamber (**A**), where trapped cells are identified by high-resolution optical system, allowing a real-time acquisition and multichannel analysis of cell population during the chip scan (**B**). Spiking experiment in which about 50–100 SKBR-3 cancer cells (from ATCC) were added to blood sample from healthy volunteers. The sample was pre-enriched with CellSearch® instrument before DEPArray™ sorting. Tumor cells and WBCs were plotted for their CK-PE (green circles) and CD45 expression (red circles), respectively (**C**). Subsequently, TCs were further selected by image analysis using CellBrowser™ software (**D**). TCs and WBCs were specifically labeled with PE anti-CK (green) and APC anti-CD45 (red) antibodies, respectively. DAPI (blue) was used to label the nuclei.

study the heterogeneity of tumor cells at the genetic level, or recovered as pools of pure CTCs.

DEPArray™ NxT allows a large degree of flexibility in the recovery with a scheme that can be design to accommodate both single-cell resolution, on one hand, and recovery of large pools on the other.

The high-quality and accuracy of DEPArray™ technology has been thoroughly validated by using immunofluorescence- and molecular-based approaches, with both spike in and real biological samples (11).

The quality and purity of cell recoveries has been first confirmed by using fluorescent microscopy. Tumor Cells (TCs)—A549 cell line—and peripheral blood mononuclear cells (PBMCs)—blood samples from human healthy volunteers, obtained with informed consent from Laboratorio di Analisi Cliniche Caravelli s.r.l—were first admixed and then recovered by DEPArray™ as single cells or in pools. The purity of recoveries was evaluated by individual expression of specific markers (Fig. 3A). Across n = 10 sorting runs, on n = 5 different machines, n = 683 cells (n = 431 TCs and n = 252 PBMCs) were recovered as single cells (n = 237) or cell pools (n = 10 recoveries, range 13–85 cells), along with n = 136 "no-cell" blank recoveries. Among cell recoveries, 681/683 (99.7%) cells were redetected using fluorescence microscopy. All recoveries demonstrated 100% purity, no PBMCs were found among TCs and vice versa, and no cells were detected among blank controls.

In addition, the purity of cell recoveries has been extensively proven by molecular analysis. In particular, whole genome amplification of single CTCs and WBCs was carried out to confirm the absence of WBC contamination by DNA finger printing (11). Besides, similar results have been published in other application domains, where direct DNA fingerprinting of individual and pools of cells collected by DEPArray™ from forensic evidence has shown achievement of 100% purity (13,15).

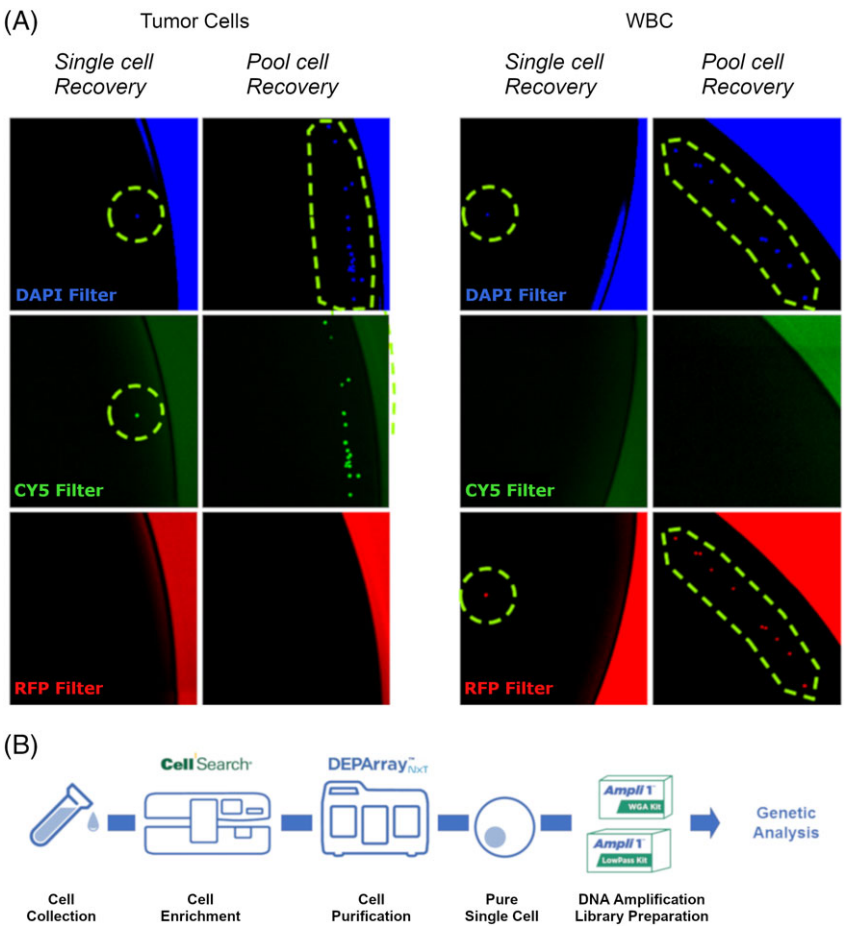

Figure 3. Representative images of DEPArray™ sorted cells (**A**). CTCs and PBMCs were labeled with CK-APC and CD45-PE respectively. Hoechst stain was used for cell nuclei. The cells were recovered as single cells or cell pool in 96-well plate. EVOS FL Auto 2 Cell Imaging System (Thermo Fisher) was used for capture the entire well surface at 4X magnification. DAPI (blu), CY5 (green) and RFP (red) filters were used for the acquisition and image analysis was carried out with Celleste Image Analysis Software (Thermo Fisher) to assess the purity of DEPArray™ recovey. Workflow for single CTC isolation and genetic analysis (**B**). CTCs are enriched (CellSearch®) from whole blood and pure single cells are isolated by using of DEPArray™ for whole genome amplification and genome analysis.

Overall, the validation assays and published results demonstrate the consistent achievement by DEPArray™ of 100% purity in the isolation of single cells (up to 96) and cell pools.

DEPArray™ System Integration in Lab Workflow

In designing the system, some key features have been implemented to ensure robustness of the workflow and automation of the process. At the end of each run the instrument automatically generate reports and executes a backup of all relevant information about the run, from set up to results. Furthermore, an optional Remote Multitask Control Workstation enables a centralized control of multiple units (up to 5) as well as centralized review of past runs in "postprocessing" mode.

Discussion

Liquid biopsy offers a noninvasive approach to follow a cancer patient over the therapeutic journey, thus, it can guide better therapeutic strategy. Liquid biopsy based on CTCs holds great promise as CTCs reflect in real-time the tumor biologic progression with all its heterogeneity. Their molecular genetic profile can be useful to identify therapeutic targets and predict response to therapies (1,6,16,17).

Different technologies have been developed to enrich and purify these rare cells (about one CTC per 10^7 WBCs per milliliter of blood) from the blood. These technologies are broadly classified in two different categories, physical-based sorting and antibody-based sorting technologies (6,7). Physical-based technologies separate CTCs based on their size- or deformability in absence of sample labeling (18,19). However, CTCs and hematopoietic cell population vary greatly in size, making it difficult to define an optimal right pore size, which could result in a loss of CTCs or contamination of WBCs. Conversely, immunological-based approaches isolate CTCs by use of specific tumor cell markers, for example, EpCAM and CK molecules, resulting in a more accurate and reproducible identification. In fact, the CellSearch® platform is the only Food and Drug Administration (FDA)-approved technology for

enrichment and enumeration of CTCs in metastatic breast, prostate, and colorectal cancers (20–23).

Regardless of the type of enrichment adopted, the contamination of WBCs mandates a second step of purification following enrichment to obtain pure CTCs for the following genetic analysis.

By combining microfluidic and microelectronic, the DEPArray™ platform exploits the principle of DEP to isolate CTCs at single cell level starting from enriched blood (10,11,22). DEPArray™ is an advanced and highly automated instrument, which complements the capabilities of blood enrichment technologies to deliver pure individual cells suitable for an accurate molecular characterization. The versatility of DEPArray™ has been largely demonstrated by different research groups (21,24–29).

Throughout the years, we have developed a standardized and reproducible workflow for the molecular characterization of single CTCs (Fig. 3B) (11,30). The CellSearch® enrichment followed by DEPArray™ purification and nonrandom *Ampli1*™ whole genome amplification (WGA) method allowed to dissect molecular signature of heterogeneous single CTCs. We have also shown that the image-based sorting allowed a correlation between the cellular morphology and the genome integrity (11), by making it possible a pre-selection of CTCs most suitable for subsequent genetic analysis.

The synergy of CellSearch® and DEPArray™ technologies combined with *Ampli*1™ best-in-class single-cell analysis is essential to detect copy number alterations (CNAs), which is a promising new area in cancer research. The importance of this analysis is reflected in the rapid increase of evidence pointing to the importance to investigate CNAs at single cell level in several settings such as small-cell lung cancer (SCLC) (24,25). In a recent work, it has been shown the importance of our CellSearch®/DEPArray™/*Ampli*1™ workflow in patient samples. By analyzing CNAs profiles of CTCs isolated from blood at baseline the authors discovered a genetic classifier for predicting whether SCLC patients will be sensitive or resistant to initial chemo-therapy treatment.

Other research groups have used this workflow reporting new insights in the mechanism of resistance to ALK inhibitors in Non-SCLC patients, identifying primary resistance due to MYC amplification (31) and evasive resistance due to MET amplification (32).

Notably, the DEPArray™ technology is not limited to CellSearch® enrichment but can be used also with other methods (21,27,28). For instance, DEPArray™ workflow has been described also for EpCAM negative-CTC population and live cells isolation. In a recent study, researchers isolated $CD44^{pos}/CD24^{neg}/EpCAM^{neg}$-CTCs from breast cancer by flow cytometry enrichment followed by DEPArray™ purification (27). Single-cell molecular analysis were carried out on different tumor subpopulation, having a different expression of neoplastic markers, and showed the heterogeneity of CTCs (27).

Another research group has reported a different method for the molecular characterization of pure CTCs from breast cancer. In particular, single-CTCs, cluster-CTCs, and cancer associated macrophage-like cells were enriched with a size-exclusion method and then purify by using DEPArray™ before the genetic analysis (21). Other applications of DEPArray™ in oncology include separation of tumor and stromal cells from FFPE (23), as well as from fresh tissue.

Conclusion

Cell-based liquid biopsy can provide information regarding tumor genetic landscape and heterogeneity both at baseline and dynamically in response to the therapy.

The CellSearch®/DEPArray™/*Ampli*1™ workflow[1] enables precise, highly automated single CTCs isolation and molecular characterization and represents a readily accessible and clinically feasible approach to make CTC an essential tool. The workflow presented finds broad application from translational research—to identify biomarkers of response and resistance—toward routine clinical application in precision oncology—to guide treatment selection both at baseline and longitudinally.

Conflict of Interest

Mariano Di Trapani, Nicolò Manaresi, Gianni Medoro are employees of Menarini Silicon Biosystems S.p.A.

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[1] The combined CellSearch®/DEPArray™/*Ampli*1™ workflow is for Research Use Only. Not for Use in Diagnostic Procedure.

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