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CellCelector™ Application Note

Automated Single Cell PCR Preparation

In-Plate Cell Sorting of Rare Sub Populations

Gentle Single Cell Transfer for Cultivation

CellCelector is protected by US patent 9,822,331 B2

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Introduction

Automated Harvesting of Cells utilizing the CellCelector

The **Cell**Celector is a flexible multiplatform system for precise isolation and secure transport of cell colonies, specific parts of colonies, tissue cluster or even single cells into a new culture environment as well as into a number of targets for subsequently performed analysis.

The instrument consists of an inverted microscope equipped with a CCD camera and a motorized stage holding the cell culture dish, a high-precision robotic arm, multiple racks for consumables and a heated holder for a reagent vial (Fig. 1). The robotic arm is mountable with different harvesting tools holding plastic tips, metal tips or glass capillaries with different diameters each fitting for the specific application. The harvesting tips are connected via a tube to a motor driven syringe pump and an automatism for system liquid re-filling. The entire unit is housed in a laminar flow hood providing a sterile atmosphere and is controlled by a personal computer via intuitive software (Fig. 2)

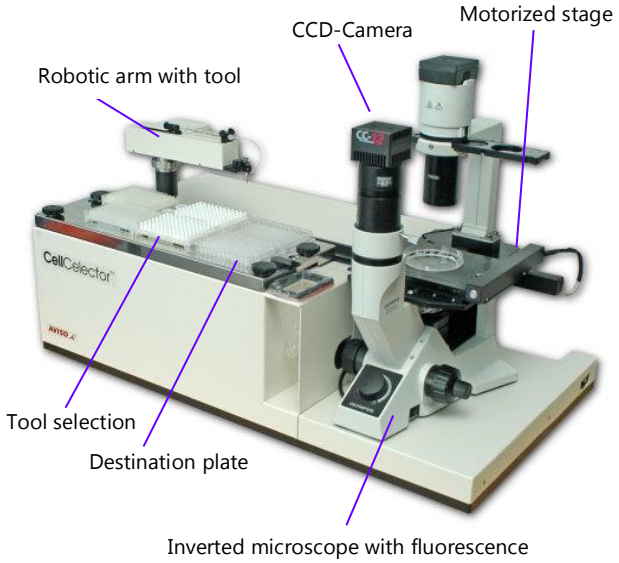

Fig.1 The **Cell**Celector - Overview

Since decades the analysis of cells at the level of genome, transcriptome and proteome as well as the creation of new clones and cell lines belongs to the focus of the modern molecular biological science. Single cell based analysis can be crucial for the molecular characterization and therefore the understanding for the biological behavior of rare cell populations like disseminated tumor cells circulating in blood from cancer patients. Modern high throughput scans of mixed cell populations require highly precise methods to detect and isolate rare cell populations or single cell. The latest state of the art techniques include a number of time-consuming single steps like cell isolation, manual pipetting and PCR-techniques performed separately each. Using the **Cell**Celector system allow the scientist to automate their workflow in a high grade or use the system case specific for precise selection and isolation of targets in an intuitive and comfortable way.

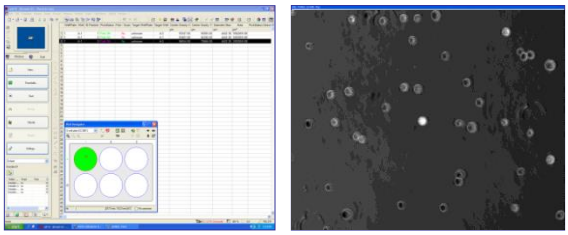

Fig. 2 Two screens of the computer: for software operation control (left monitor) and for real-time imaging or selection of single cells (GFP-positive HT29 cell) on-screen via mouse click (right monitor)

The reliable analysis of single cells regarding their gene expression pattern as well as the generation of new cell lines depend on gentle harvesting conditions. In this study we implemented the **Cell**Celector technology to automatically detect and isolate a number of cell types of different origin.

Technology and Work Flow

1. Scan & Imaging

The culture dish with the region of interest is scanned automatically by a high resolution camera employing the motorized stage (Fig. 3 left). The entire collection of single images during the scan is combined by the software into an overview image (Fig. 3 right). Integrated fluorescence filters can be very useful in terms of immunochemical labeling and staining and allows the user in-plate cell sorting before picking.

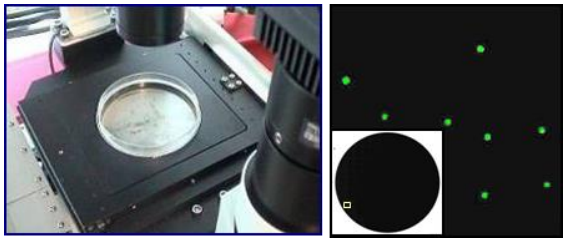

Fig. 3 Scanning process of a petri dish with cell culture on the motorized stage (left); overview image with blow up area (right). The green dots represent detected single cells.

2. Selection & Targeting

According to selection parameters (size, diameter and shape factor of the cell etc.) predefined by the user the software detects and selects the targeted cells or colonies (Fig. 4). Selection can be done using a great variety of analysis methods provided by the microscope (fluorescence, bright field, phase contrast) and the Cell*D software providing 3D imaging, overlays and movies. An overlay of phase contrast and fluorescence image can be created.

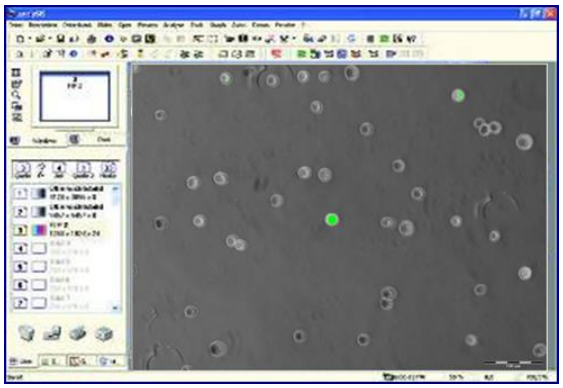

Fig. 4 Cell culture in the imaging software for selection of a specific target region or cell

3. Automatic Harvest

An application-specific harvesting module takes the cells or colonies up and the robotic arm automatically transfers them to the destination plate according to a user-defined chart (Fig.5).

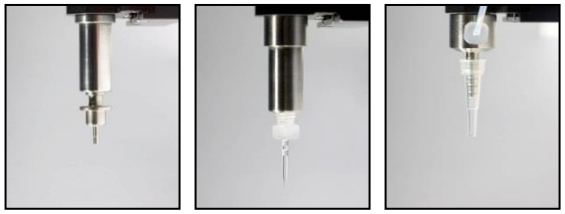

Fig.5 Different tools for automatic cell and colony transfer Moduls (le. to ri.): Scrape Module, SingleCell Module, MC Module

The ScrapeModule is convenient for transfer of whole or parts of cell colonies. Single cells can be taken up most sensitive by the SingleCellModule using glass capillaries with a diameter of 20 to 220 µm (Fig. 6). The MC-Module uses plastic tips with a larger diameter for harvest of Hybridoma colonies out of methylcellulose medium.

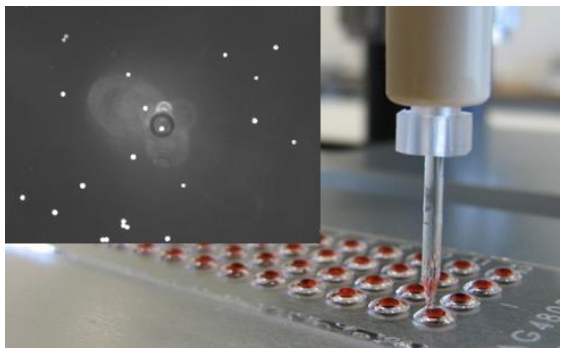

Fig. 6 SingleCellModule for isolation of single cells during the aspiration of a GFP positive HT29 cell (small image) and its preparation on AmpliGrids for single cell PCR analysis (big image)

4. Documentation

In order to keep the whole process reproducible and documented an image of each cell or colony before and after harvesting is stored in the database and assigned to the deposit position in the destination plate (for examples see chapter "Precise isolation of single cells"). That allows for comparing growth progression of colonies or cells and later check-up during downstream processes (PCR, Arrays etc.).

Software

The **Cell**Celector Software (Fig. 7) contains numerous features for individual analysis of cells and cell colonies either automatically or on-screen. The software combines imaging facilities (camera control, fluorescence, overlay etc.) with the robot control for the cell harvest.

It is possible to select specific cells of interest on-screen. Driving the microscopic motor stage allows for searching within the culture dish for target cells and to mark them by mouse click, either for instant picking or for collection of multiple targets into a picking list.

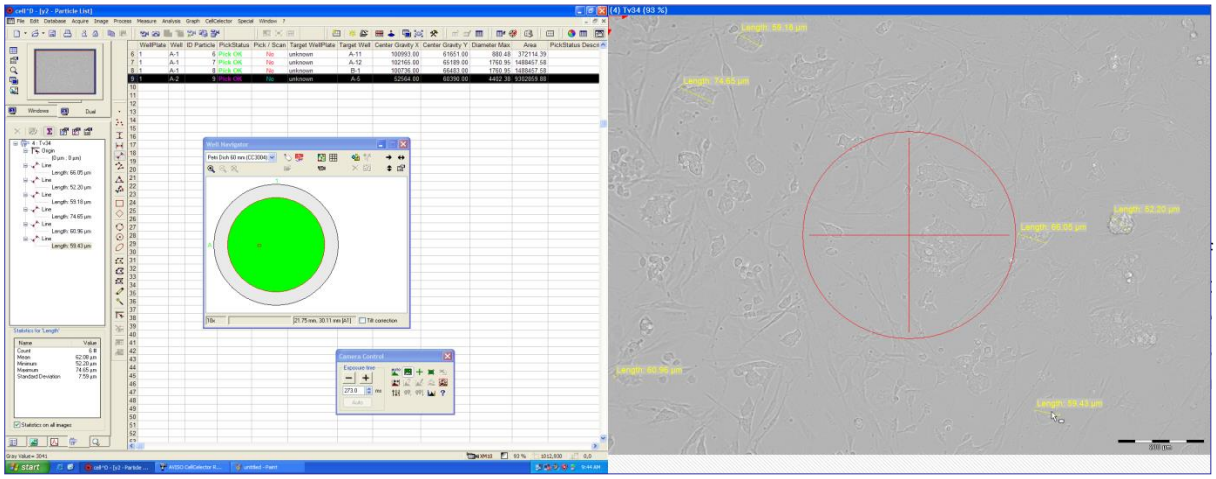

Fig. 7 Screenshot of the software showing software control with well navigator (left) and microscopic image of mouse tumor cells with measured cell diameter.

The **measurement bar** provides several tools for measurement of cells, colonies or structures on-screen like diameter, area, distance to adjacent cells etc. (Fig. 8). It is helpful for identification of targets as well as for the right choice of capillary diameters.

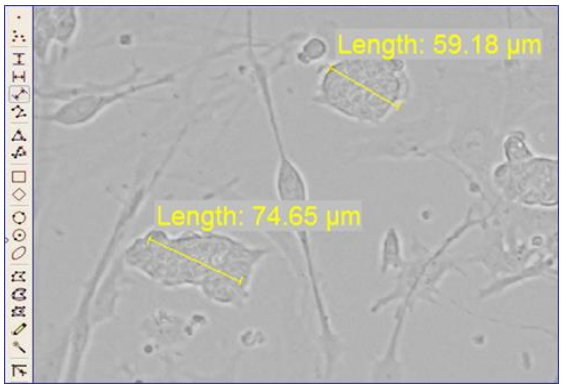

Fig. 8 Measurement bar (left) and close image from screenshot above showing application of the measurement tools on-screen (right)

The **well navigator** (Fig. 9) indicates where the camera focuses on. Wells can be addressed for cell harvest and focus by mouse click and will be shown in green color. The microscope motor stage is connected to the navigator and will be driven to the selected point of view. Special formats of plates and dishes can be easily configured by the user and saved for later use.

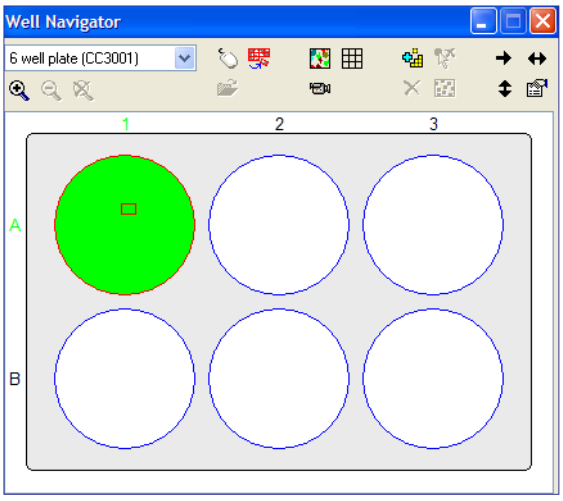

Fig. 9 Well navigator showing a 6 well plate

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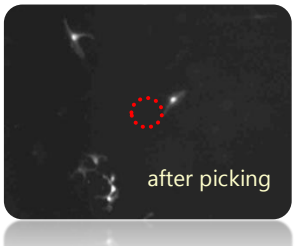
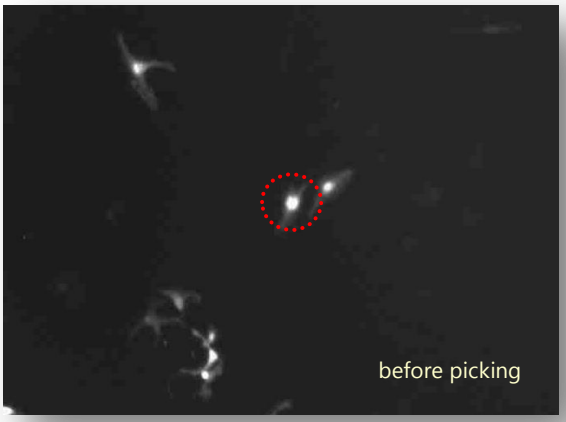
Precise Isolation of Single Cells

Mesenchymal Stem Cells

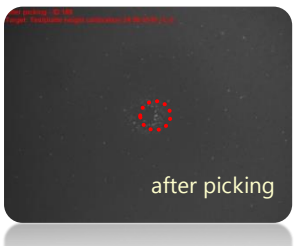
Mesenchymal stem cells (MSCs) are progenitor cells of the connecting tissue. MSCs obtained from human bone marrow have the ability for extensive self-renewal and clonal expansion, as well as the capacity to differentiate into various tissue types and to modulate the immune system. They can differentiate into various cell types of the connective and supporting tissue e.g. osteocytes, chondrocytes, myocytes and many more.





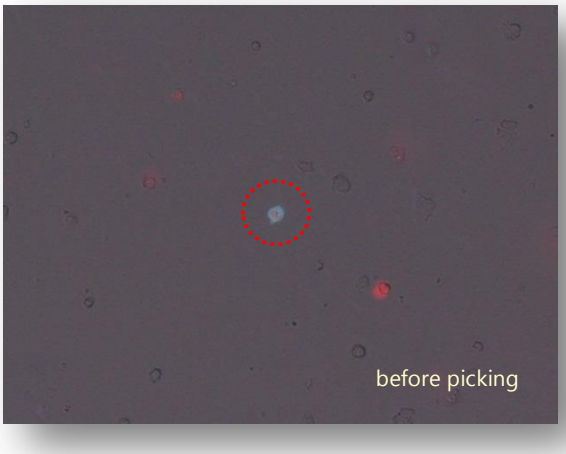
Murine B Cells/Plasma Cells

B cells stimulated with bead coupled epitopes turns to antibody producing plasma cells. After detection the secreted epitope specific antibodies by fluorochrome coupled antibodies a halo around the active plasma cell appear. After isolation the epitope specific plasma cell the antibody coding genes are analyzed and cloned.



Human Circulating Tumor Cells

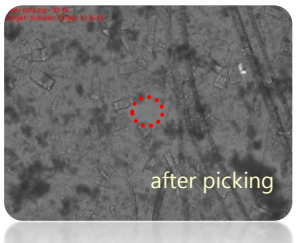
Epcam positive cells were enriched from blood of patients with breast cancer. The isolated disseminated tumor stroma cells (Epcam-positive; PI-negative) were analyzed for its gene expression pattern.

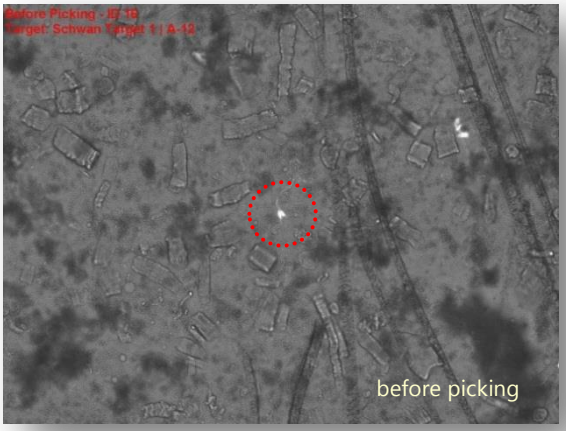


Precise Isolation of Single Cells

Schwann Cells (Axolotl)

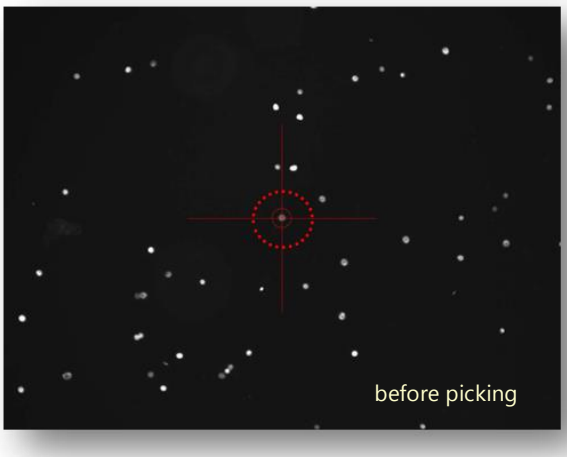
In this evaluation GFP tagged Schwann cells prepared from food of Axolotl were isolated. The tissue was mashed and resuspended in an adequate dilution. Schwann cells could be easily detect via a combined Bright Field and Fluorescence Illumination and isolated precisely from the debris.





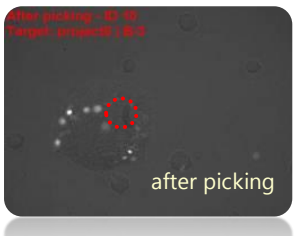
Colon Carcinoma Cells (HT29 Cells)

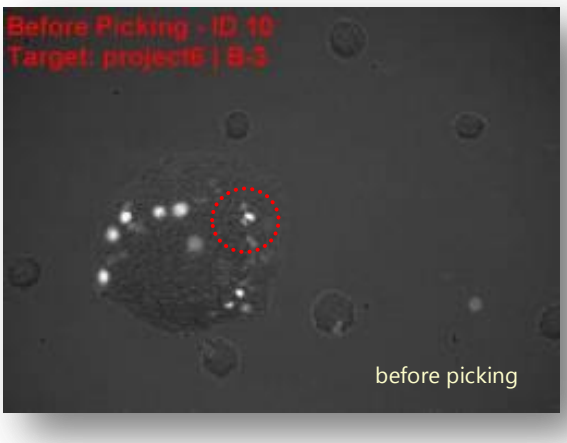
The Human colon adenocarcinoma grade II cell line HT29 is an often used model system in cancer research. Here we evaluate the conditions (dilution, surface of culture plates or picking settings) to automate the isolation of circulating tumor cells.



GFP-Positive Single Cells from Mosaic Colonies

In this experiment we successfully evaluate the precise isolation of single cells from cell colonies.





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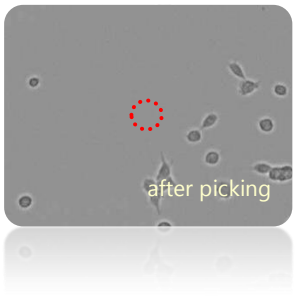
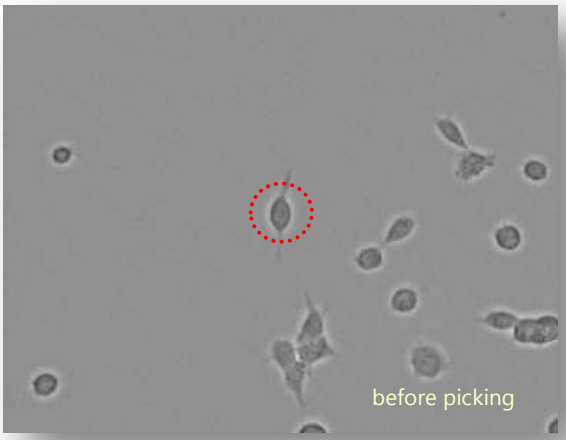
Precise Isolation of Single Cells

Human Embryonic Kidney Cells (HEK)

HEK cells have been widely-used in cell biology research for many years. There are easy to grow and used for transfections in research and for therapeutic protein production in the bio-technological industry.





Human Sperm Cells

The genomic analysis of human sperm cell is of special interest in the field of criminal forensic.

Fungal Spores

In this case spores of Sordaria macrospora were picked. In other experiments spores germinating in agar were isolated.

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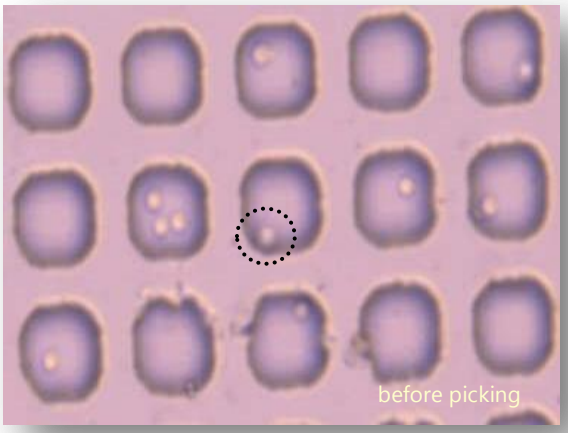
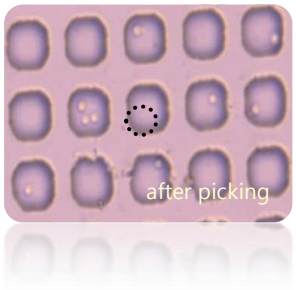
Special Application in Precise Isolation of Single Cells

Automated Recovery of Single Cells Identified by Microengraving

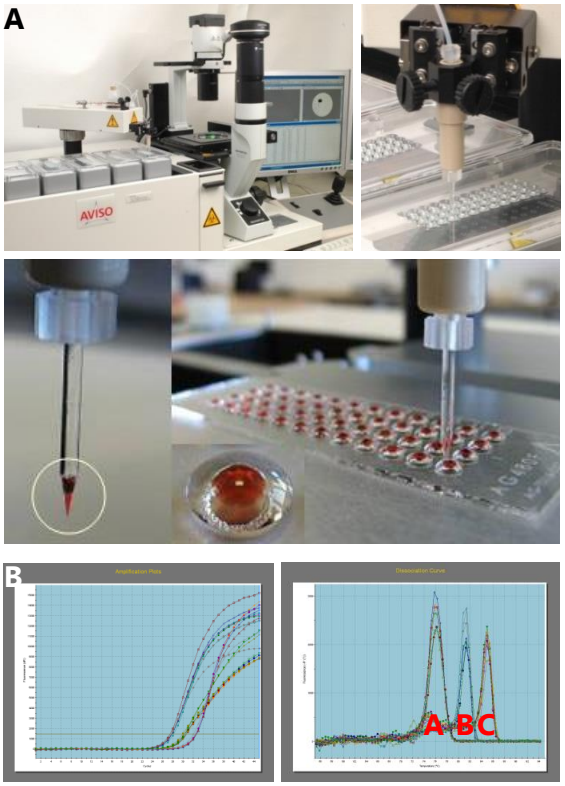
A mixture of two mouse cell lines (12CA5 and HYB099-01) was distributed on micro scale Polydimethylsiloxane (PDMS) slides carrying 84672 cuboid microwells with an edge length of 50 µm. Using the unique microengraving technology a subpopulation from a cell mixture was identified and isolated with the **Cell**Celector as single cells from the micro well. Using microengraving technology single cells can be analyzed for many criteria by high throughput screening before isolation of the cell sub population of interest.





Automated Detection, Isolation and Genetic Analysis of Single Circulating Tumor Cells

CD90 positive cells from patients suffering breast cancer were detected using several fluorescence criteria and isolated using glass capillaries with an inner diameter of 50 µm. Just before picking, PCR mix was aspirated in the needle. The picked cell was released on anchor positions at the surface of Ampligrids (see right figure on the right), the bottom of PCR plates or the lid of Eppendorf cups. During releasing the cell was mixed with the PCR mix and covered with oil to prevent evaporation in a unique single step manner (see right figure A). Subsequently performed RT-PCR and q-PCR for 3 genes revealed the reliability of picking and transfer for 8 picked single cells. The right image B displays the (dR) values (left) and melting curves (right) for genes A, B and C from 8 independent single cell reactions.



Selection of Target Single Cells

The automatic detection of single cells requires a set of target specific characteristic parameters. The basic detection process is working with grey or color values. Setting a range of grey values (by definition light and dark color thresholds fitting for the cells of interest and visualized as green colored areas on the reference image) can be sufficient to separate the target cells from the background – especially for cells labeled with fluorescence markers (Fig. 10). After scanning the detected cells are displayed as overview map (Fig. 11) and listed in a data sheet.

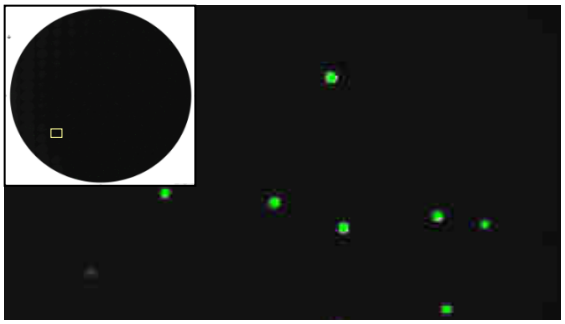


Fig. 11: Overview map (top left) after scanning GFP-expressing H9 cells and enlarged area marked by the rectangle.

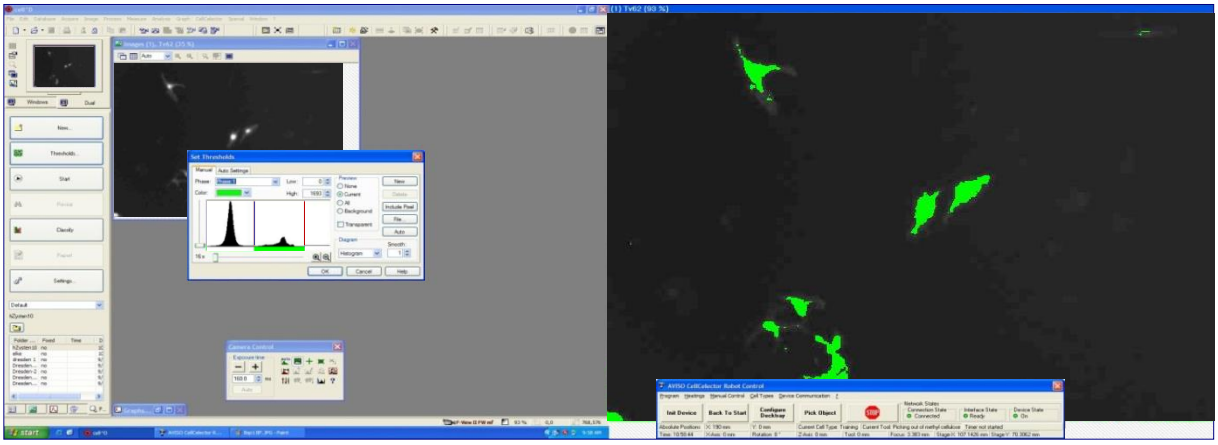


Fig. 10 Screenshot of setting grey value thresholds for mesenchymal stem cell detection

Changing the range of grey values (Fig. 12 A and B) allows the user to specify the areas to detect (Fig. 12 D and E). By combining the grey value based obtained signals (Fig. 12 D and E) with additional parameters like size, diameter, shape factor, sphericity etc. (Fig. 12 F) it is possible to increase the quality of the scanning results significantly (Fig 12 G and H).

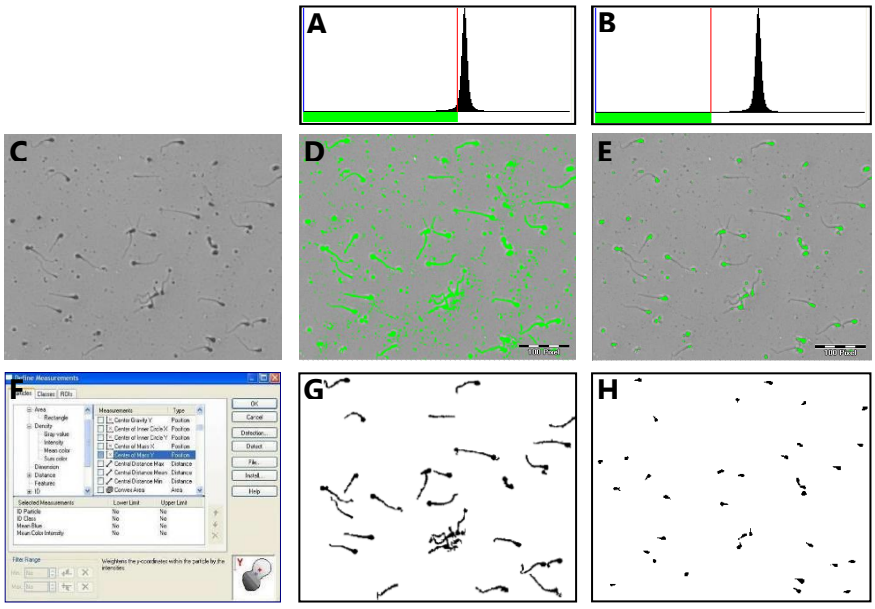


Fig. 12 Setting parameters for scanning human sperms. Different grey value thresholds (A & B) detect different parts of the sperms (D & E) from the original reference image (C). Additional parameters (F) improve the scanning result (G & H).

To improve the scanning result further it can be useful to process the original reference image as first step while scanning. As shown in Fig. 14 single plasma cells were detected secreting antibodies which are visualized by fluorochrome labeled antibodies (Fig. 14 A) and detectable only as cloud-like conglomerate (Fig. 14 B) holding a number of picking positions calculated for each dot. Using morphological filter features (Fig. 13) the dotted halo surrounding the cell can be processed into a consistent area (Fig. 14 C) which is detectable as homogeneous colored area (Fig. 14 D) with a single picking position in its central part.

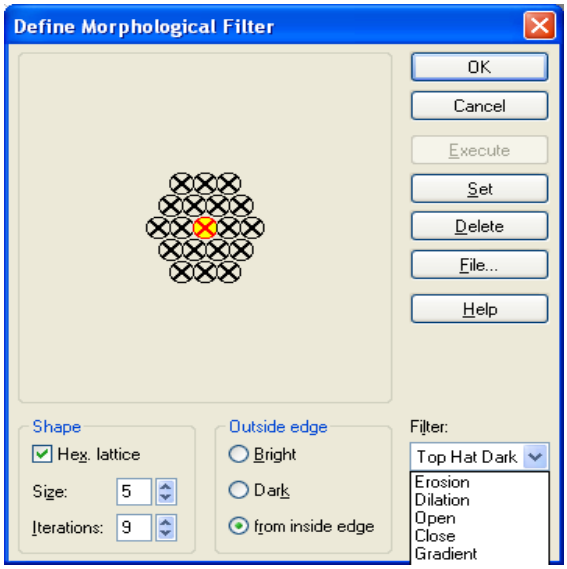


Fig. 13 Dialog window to define morphological filter settings

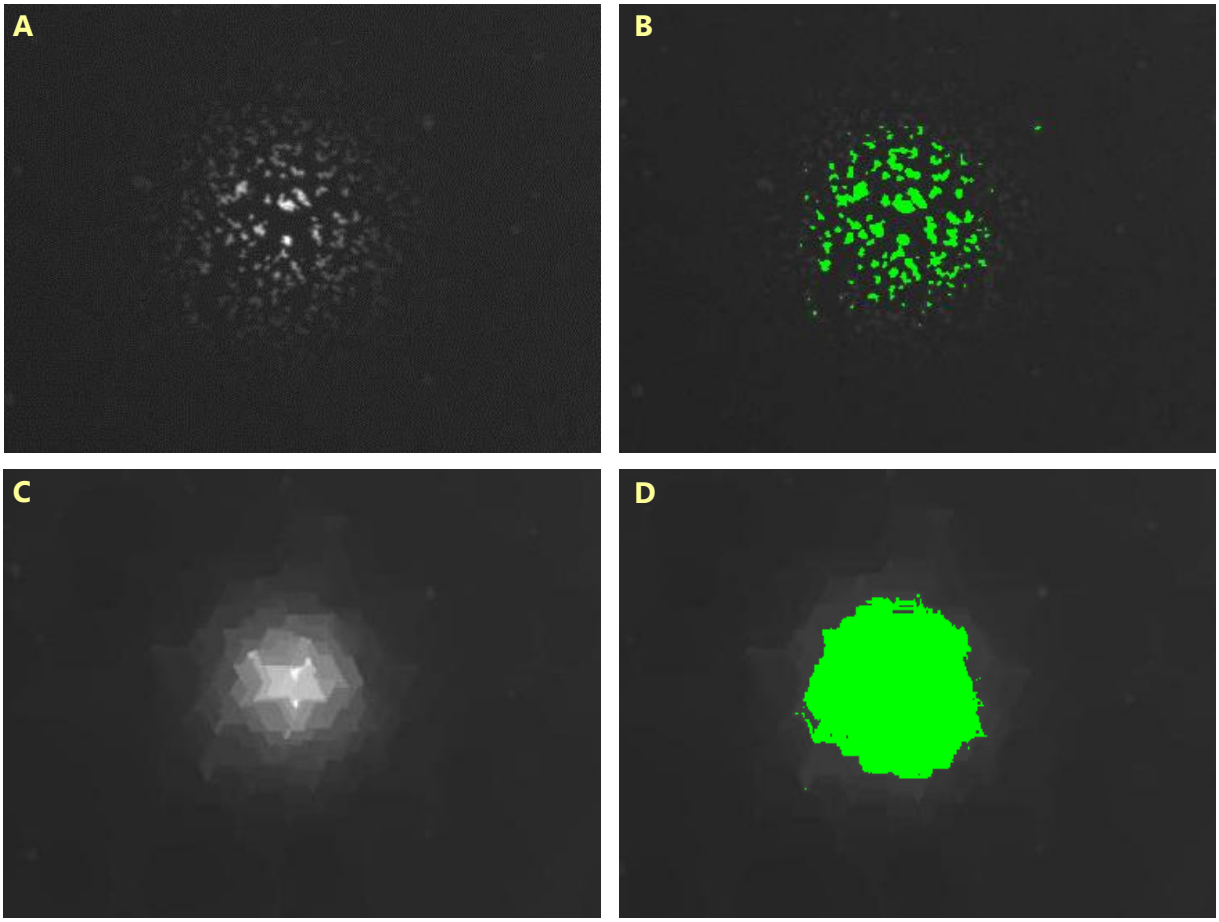


Fig. 14 Detection of antibody secreting plasma cells. The plasma cells are located within the central part of the detectable dotted halo. Original image (A) and its detectable areas (dotted halo (B); processed image (C) and its detectable homogeneous area (D).

Conclusions

Efficiency

The **Cell**Celector is an efficient and highly selective tool for a safe transfer of single cells and cell colonies without interfering with important properties of the cells such as pluripotency and viability. Several experiments proofed that an automated process can improve the quality of transferred single cells or cell colonies and allow to set standardized condition for the entire cell picking process. Of special importance is the cell sorting capacity in the culture plate directly to target cell subpopulations before picking the cells.

Sensitivity

An assessment of pluripotency-associated markers and differentiating cells after automated picking and replating for several times confirmed their pluripotency status and a lower number of dead cells compared to picking by hand. Several parameters can be combined individually to apply the right and most gentle resolving aspiration force. By that and using the heatable destination positions (37°C) the mechanical stress is reduced and the viability of cells after transfer is increased. Highly precise tools allow for a safe transfer of even single cells provide new possibilities in stem cell research. With a reproducibly small amount of aspiration (below 0.1 µl) quantitative single cell RT-PCR and PCR analysis becomes a standard method.

Flexibility

The **Cell**Celector also enables the scientist to select cells precisely according to their state of differentiation using different fluorescence excitations and markers at the same time. Hence, the integration of a state-of-the-art microscope which is widely used in laboratories provides an innovative and time-saving combination of various analysis methods and a direct transfer into a new culture environment or wells for further genetic analysis (PCR). For working with primary cells and tissue the **Cell**Celector and the ALS Incubator **Flow**Box (Fig. 16) are recommended since physiological conditions like temperature and CO_2 atmosphere can easily and precisely be adjusted.

Security

When working with cells determined for transmission to patients contamination with pathogens is an important issue. The complete automation of the picking process decreases the need of manual intervention (dish positioning) and therefore increases the security of valuable cell material from contamination with retroviruses or other pathogens.

The **Cell**Celector is placed under a sterile hood and resistant against intense surface sterilization using Ethanol and UV-light.

Fig.15 The **Cell**Celector can be placed in a flow box for increased safety of cell cultures

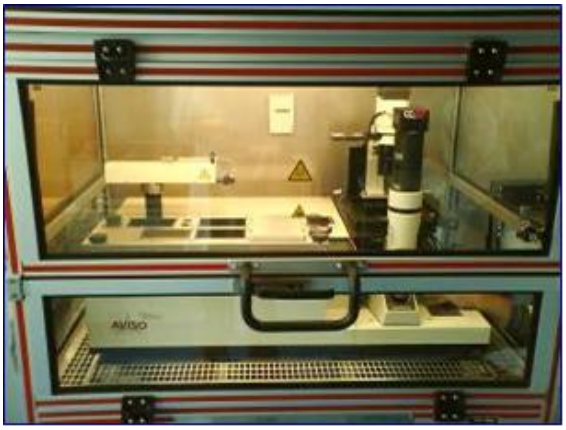


Fig. 16 The **Cell**Celector is placed in the ALS Incubator **Flow**Box with high CO_2-atmosphere and heated environment (37°C), especially useful for long-term experiments and primary cell cultures.

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ALS Automated Lab Solutions GmbH headquarters in Jena, Germany

ALS Automated Lab Solutions GmbH is located in Jena, a dynamic city famous for microscopy and material science. ALS is a specialist for the development of innovative technological solutions for cell biology research and molecular biology. ALS lifts cell culture to a new level of choice and control on the leading edge in cell biology, cell therapy research, regenerative medicine and drug discovery. With automation and standardization of laborious manual procedures, ALS supports science and research for more efficiency and the creation of new methods for the science of tomorrow.

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