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CellCelector™ Application Note

Automatic Stem Cell Transfer

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

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Introduction

Automated Harvesting of stem cells utilizing the CellCelector

The AVISO **Cell**Celector from ALS Automated Lab Solutions is a flexible multiplatform system for precise isolation and secure transport of cell colonies, specific parts of colonies 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 motorised stage holding the cell culture dish, a high-precision robotic arm, multiple racks for consumables and a heated holder for a reagent vial. 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 (see chapter software). [4]

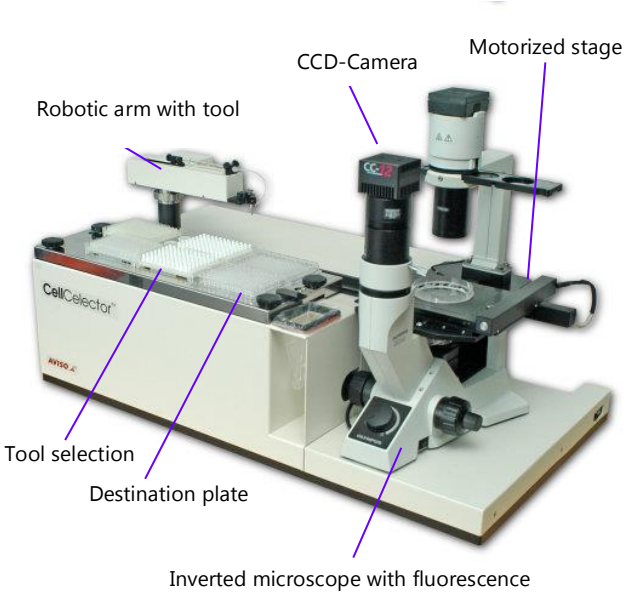


Fig.1 The **Cell**Celector - Overview

The ability of **human embryonic stem cells (hESC)** to differentiate into specialised cells of all three germ layers (pluripotency), their capability for unlimited cell division (self-renewal) and their amenability to genetic modification provide fascinating prospects for the generation of genetically modified human cell lines for biomedical and pharmaceutical research.

Recently, **induced pluripotent stem (iPS)** cells have emerged as an additional source of pluripotent cells, which can be derived from adult somatic tissues (Takahashi K *et al.*, 2006, 2007).

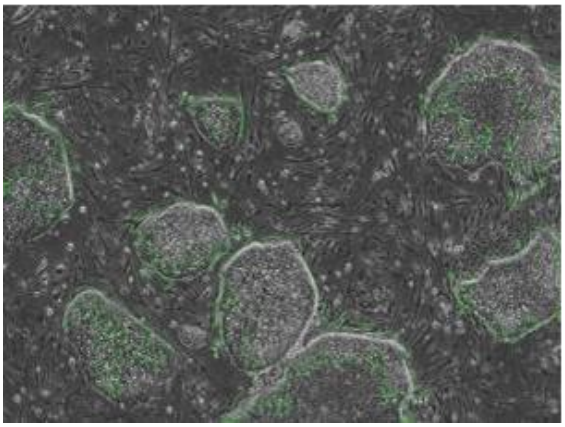


Fig. 2 Colonies of human ES cells (kindly provided by Prof. Dr. Brüstle, Life&Brain, Bonn, Germany)

Both, the selection of successfully engineered hESC and the derivation of iPS cells depend on the harvesting of individual stem cell colonies, which are subsequently further expanded to obtain homogenous cell lines. In this study we implemented the CellCelector™ technology to automatically detect, isolate and propagate human ES cells as well as murine iPS cells.

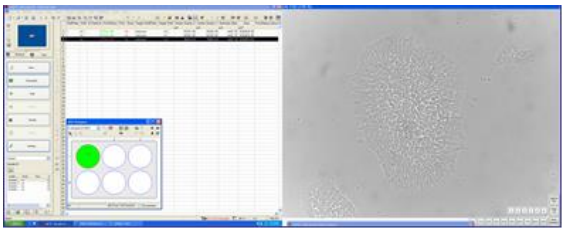


Fig. 3 Computer with two screens: Left monitor for software operation control, right monitor for real-time imaging and manual on-screen selection by mouse click

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. 4). The entire collection of single images of the scan is combined by the software into an overview image. Integrated fluorescence filters can be very useful in terms of immunochemical labelling and staining and allow the user to do multiple fluorescence based cell analysis inside the culture plate before picking.

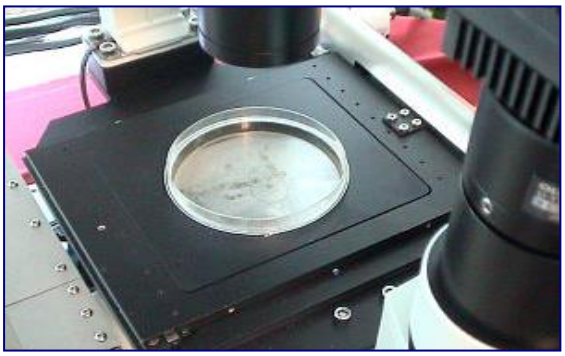


Fig. 4 Scanning process of a petri dish with cell culture on the motorized stage

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. Selection can be done using the 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.

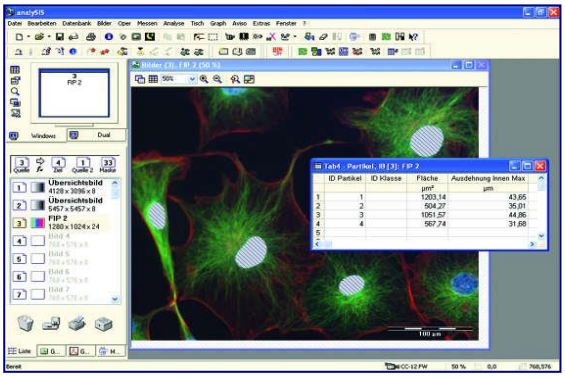


Fig.5 Cell culture in the imaging software for selection of a specific target region or cell

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).

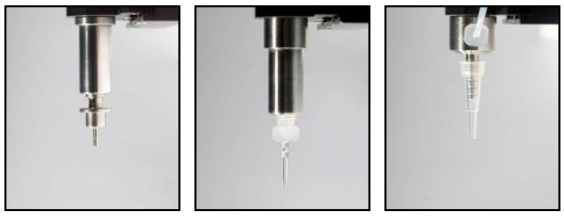


Fig.6 Different tools for automatic cell and colony transfer (left to right): Scrape Module, Single Cell Module, MC Module

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

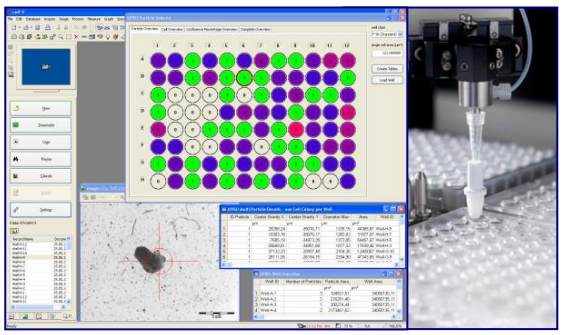


Fig. 7 Online documentation of original position, destination well and colony shape parameters in the software (left), Clone tip module for local trypsinization of cells (right)

4. Documentation

In order to keep the whole process reproducible and documented an image of each cell or cell colony before and after harvesting is stored in the database and assigned to the deposit position in the destination plate (Fig. 7). 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 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 entire or specific areas out of cell colonies on-screen. Driving the microscopic motor stage allows for searching within the culture dish for target cells and then marking them by mouse click.

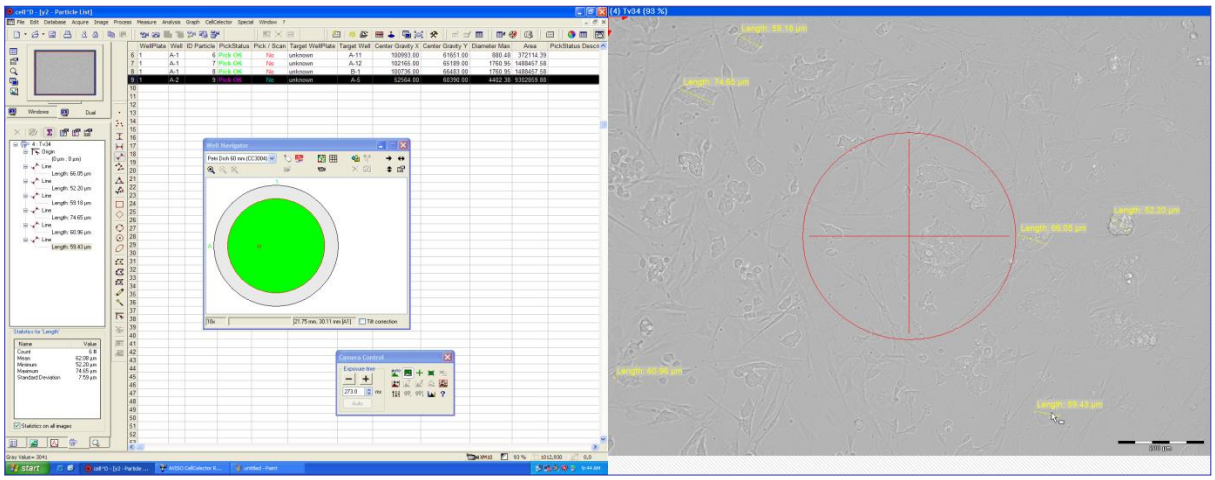
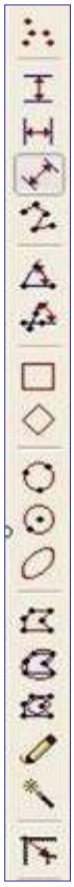


Fig. 8 Screenshot of the software showing software control with well navigator and (left) mouse tumour cells with diameter figures.



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

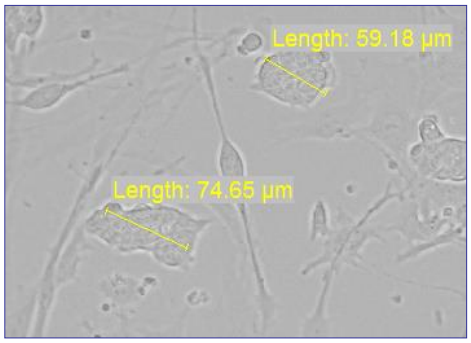


Fig. 9 & 10 Measurement bar (left) and close up image from screenshot above showing application of the measurement tools on-screen (right).

The **Well navigator** 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.

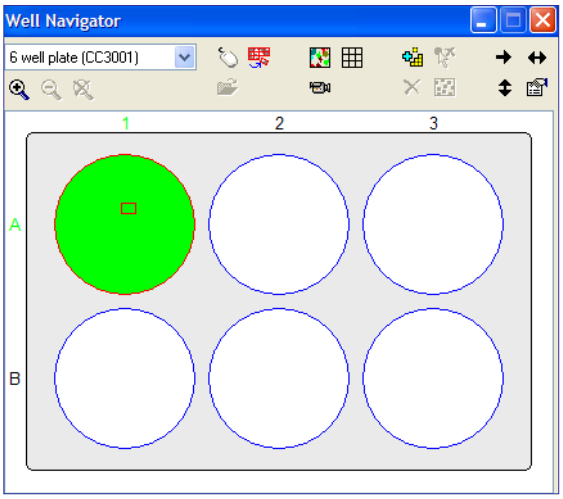


Fig. 11 Well navigator showing a 6 well plate

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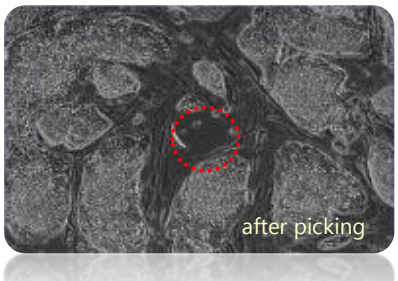
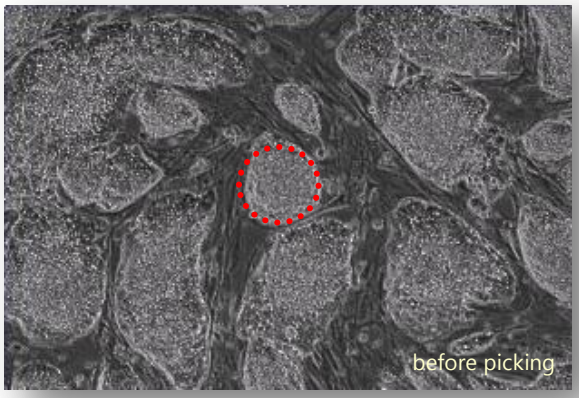
Precise Isolation of Stem Cells

hESC Colonies from Feeder Cell Layer

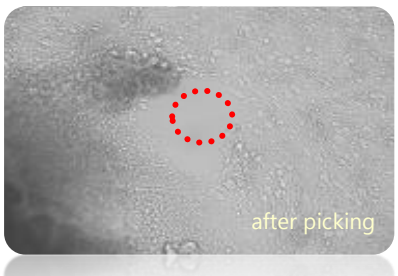
It is a commonly used method to co-cultivate undifferentiated stem cells with feeder cells, mostly fibroblasts in order to provide an environment that keeps stem cells stable and viable. However, the isolation of stem cells without transferring feeder cells requires refined skills and can be done with the **Cell**Celector more precisely.

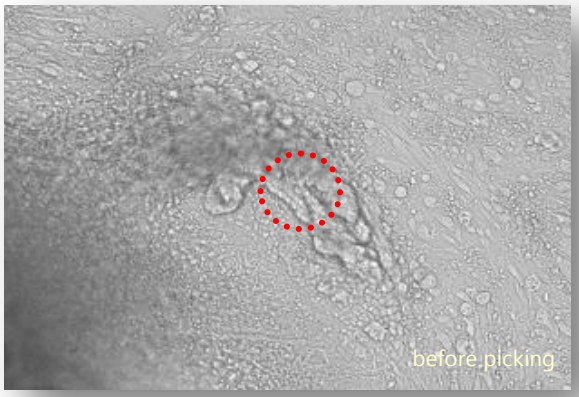




Differentiated Parts out of hESC Colonies

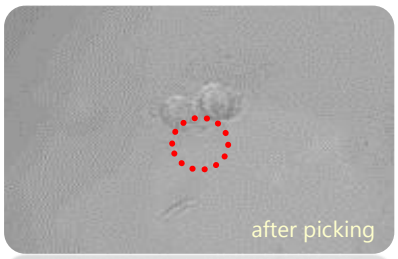
In this experiment we evaluated specific parts of already differentiated areas of the stem cell colonies live on the screen. A subjective recognition on screen was more useful due to the special characteristics of the cell layer. Differentiated cells form a tissue-like layer with strong cohesion.

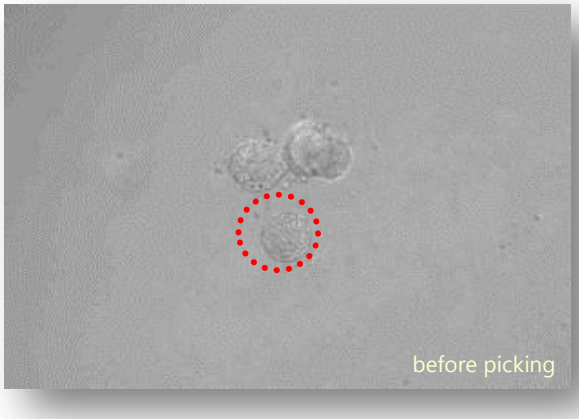




Embryoid Bodies (EB)

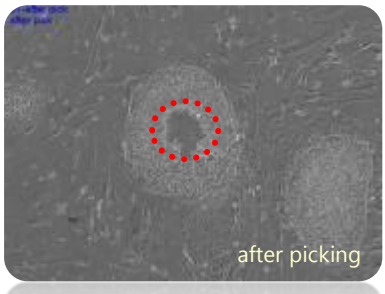
Stem cell colonies can be cultivated three-dimensional in highly viscose media like methylcellulose or Matrigel. So called EBs can be used to study organ formation at a very early stage

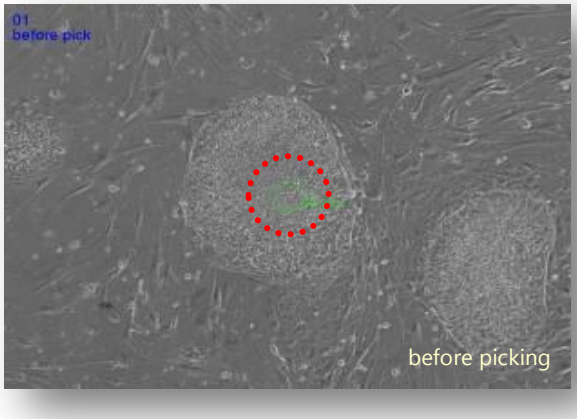




Isolation of Small Parts of a hESC Colony

A precise excision of special parts with a colony can be done by using the SingleCell Modul. The size of the installed glass capillary is being shown in the image on-screen.





Neural Stem Cells (NSC)

NSC are self-renewing, multipotent cells that can develop into various cell types of the brain and the spinal cord.

Mesenchymal Stem Cells (MSC)

Mesenchymal stem cells 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 Osteozyten, Chondrozyten, Myozyten and many more.

Selection of Target Cells and Colonies

The automatic recognition of cells and colonies require 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 (Fig. 12) – especially for cells labelled with fluorescence markers.

By clicking with the mouse to positions within the map the microscope motor stage automatically moves to the cell or colony of interest which will be shown in real-time on the screen. After second evaluation by the user parameters can be refined or the harvest can be initiated.

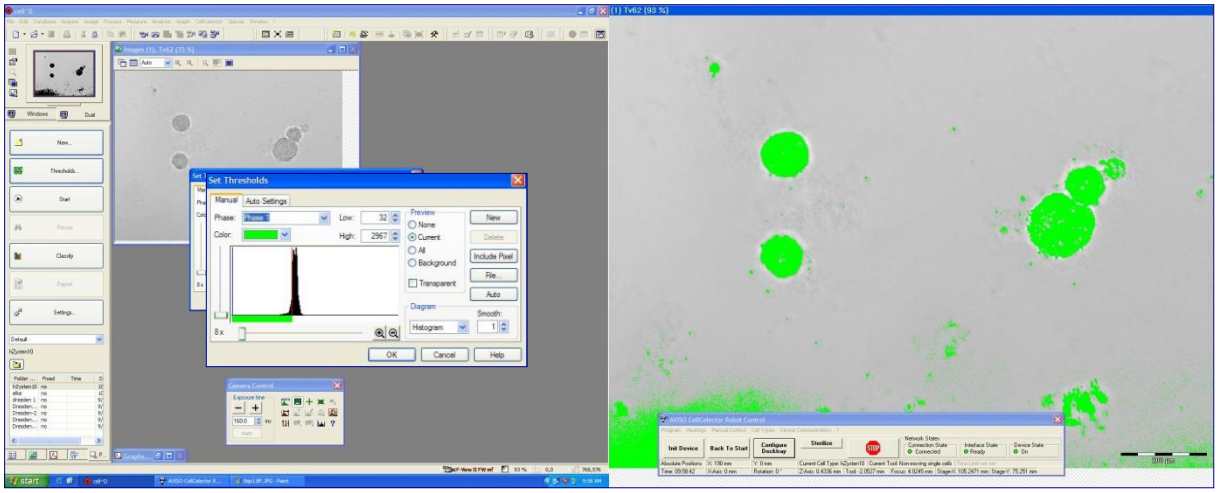


Fig. 12 Screenshot setting thresholds for target identification (left) and detected cell colonies displayed in green color fitting in that range (right)

After the scan the detected cells are displayed as overview map and listed in a data sheet.

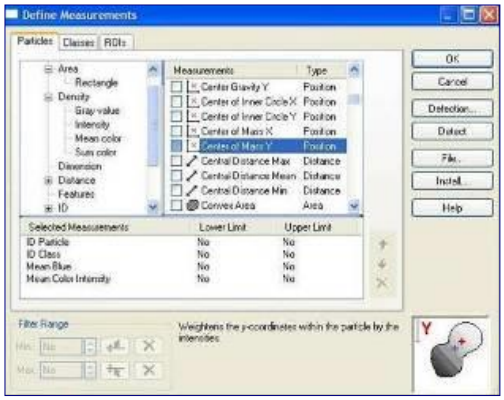
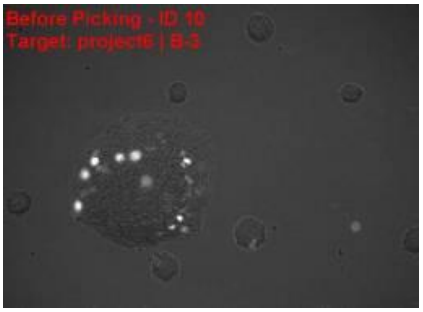


Fig. 13 Screenshot of a list of parameters usable for the scan



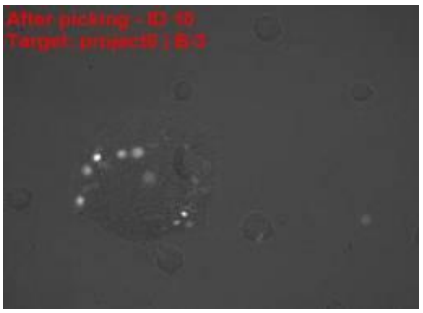


Fig. 14 & 15 Before (left) and after (right) picking of a small fluorescent cell area out of a mosaic colony using overlay modus of fluorescence and phase contrast image.

Higher Rate of Cell Survival after Transfer

Transfer of colonies is often a stressful procedure to the cells resulting in a great number of dead cells influencing their living neighbours [2]. It can have distinct effects on the phenotype of stem cells and cells quite readily begin to differentiate. Therefore, it is crucial to use a sensitive mechanical transfer method causing the least amount of destroyed cells [1].

Efficiency of the transfer process was determined by counting the number of hESC clusters directly after transfer into a 96-well plate. In the result, both numbers are in the same range. Quantification of replated colonies was performed 4 days after transfer (Fig. 16).

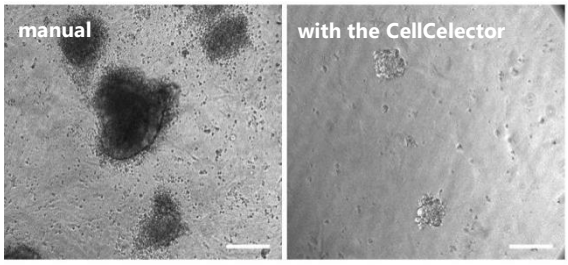


Fig. 16 Phase contrast image of depicted hESC clusters after manual transfer with a 100 µl pipette tip (left) and a 220 µm glass capillary (right; scale bar 200 µm) with the **Cell**Celector

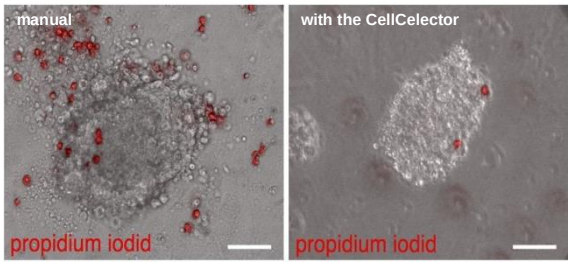


Fig. 17 Propidium Iodid staining for imaging dead cells after manual (left) and automatic transfer with the **Cell**Celector (right)

Propidium iodide (PI) incorporation (Fig. 17) was used to assess cell survival of manually (left) and automatically (right) picked hESCs after transfer. Phase contrast images of those hESCs were merged with the corresponding fluorescent images of PI staining (scale bar 50 µm).

The shape and structure of a colony can also provide important information about the current state. A phase contrast image (Fig. 18) of a representative colony was taken 3 days (left) and five days (right) after automated passage into a new culture dish. The colony showed normal growth.

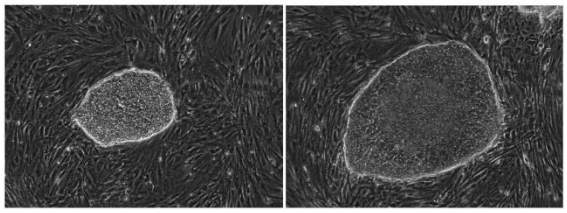


Fig. 18 hESC cell colony in the phase contrast; (right) 3 days, (left) 5 days after transfer

DNA content as well as DNA synthesis is a reliable indicator of the cell density, activity and viability of the transferred colony and therefore testifies the reaction of the cells to the method of transfer.

BrdU is a nucleoside analogue (thymidine). Proliferating cells with DNA *de novo* synthesis will integrate it instead of dTTP. Conjugated antibodies were used to label these cells and thereby providing visual evidence of cell division

BrdU incorporation was analyzed (Fig. 19) by FACS using the fluorescent dye Alexa 488 to measure BrdU and Hoechst to determine total DNA content. Gates were set for cells in the S-phase, G1-phase and G2-phase to determine the percentage of proliferating cells. In the result it turned out that a passage using the CellCelector is slightly better tolerated by the cells than the manual way.

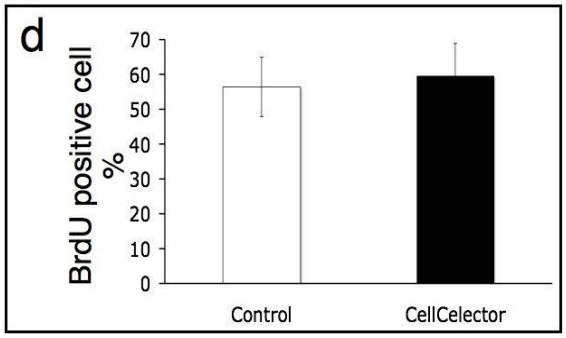


Fig. 19 BrdU positive cell counted after manual transfer of hESC and using the AVISO **Cell**Celector.

hESC maintained Pluripotency, Growth Characteristics and In Vitro Differentiation Potential

Most cell colonies do not tolerate multiple transfer very well. Hence, it was tested how stem cells react in regards of their state of pluripotency. **Immunochemical expression analysis** of typical markers as well as the search for genetic aberrations are reliable methods to elucidate the effects of transport of the cells. Typically, particular proteins playing a pivotal role in maintenance of pluripotency or determination of cell fate can be tagged by antibodies coupling fluorescent dyes easily detectable by the integrated microscope with imaging software.

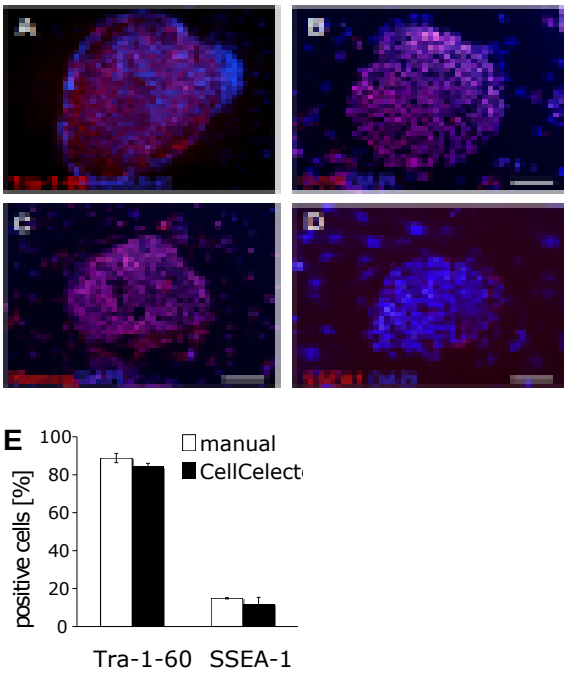


Fig. 20. (A-D) Immuncyto-chemical stainings for the pluri-potency-associated surface marker Tra-1-60 (A), the pluripotency factors Oct4 (B) and Nanog (C) and SSEA-1, a surface marker for differentiated human cells (D). Primary antibodies were visualized with Alexa-555-coupled secondary antibodies. Cell nuclei were counterstained with Hoechst or Dapi. (E) Quantification of cells expressing the pluripotency-associated marker Tra-1-60 and the differentiation marker SSEA-1 was done by FACS analysis .

After multiple passages (3x) with the CellCelector hESCs maintained expression of pluripotency-associated markers Tra-1-60, Tra-1-81, Oct4 and Nanog (transcription factors typical for hESC) as shown by immunocytochemistry (Fig. 20), FACS analysis (Fig. 21 upper part) and western blot (Fig. 21 left down).

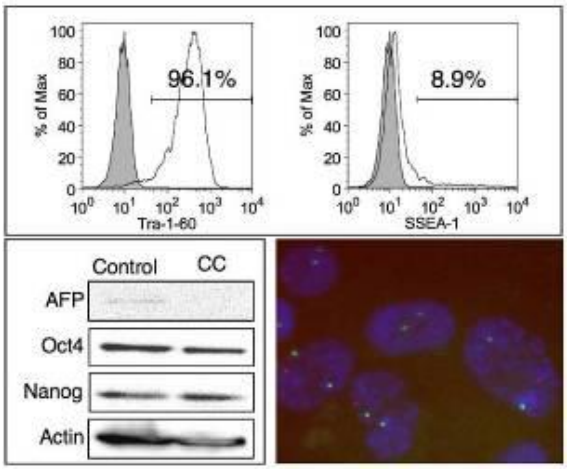


Fig. 21. FACS analysis and Western Blot (le. Down) of pluripotency markers; (ri, down) FISH for chromosome trisomy screen

Expression levels are comparable to those of conventionally propagated hESCs. Fluorescent *in situ* hybridization (FISH) was performed for chromosomes 12 and 17 to screen for alterations (trisomy) often observed during long-term propagation of pluripotent hESCs (Fig. 21). Multi-germlayer differentiation potential of automatically picked and transferred hES cells was not affected. Upon formation of embryoid bodies (EB) they gave rise to endodermal (AFP), ectodermal (Cytokeratin) and mesodermal (Desmin) cell types as shown in Fig. 22.

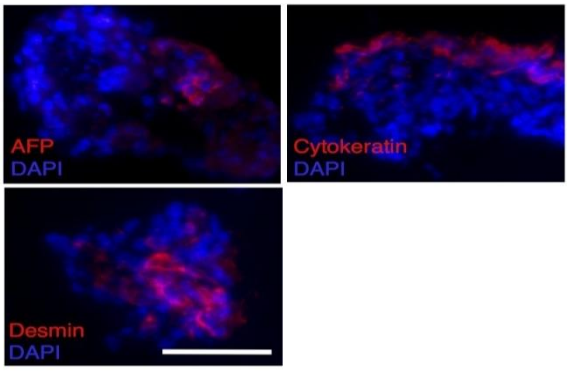


Fig. 22 Cell types were visualized by immunocytochemical analysis of plated EBs using Alexa-555 coupled secondary antibodies, Cell nuclei were counterstained with DAPI.

Conclusions

Efficiency

The **Cell**Celector is an efficient and highly selective tool for a safe transfer of stem cells and 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 cells or colonies. But automation also becomes helpful when larger quantities of hESCs are needed for experiments [1] or stem cell banks.

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. 24) are recommended since physiological conditions like temperature and CO2 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 necessary manual intervention (dish positioning) and therefore increases the security of valuable cell material from contamination with retroviruses or other pathogens [2]. The **Cell**Celector is placed under a sterile hood and resistant against intense surface sterilization using Ethanol and UV-light.

Fig. 23 The CellCelector can be placed in a flow box for increased safety of cell cultures

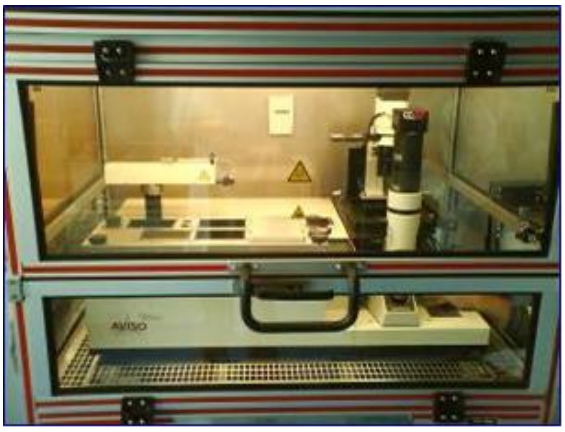


Fig. 24 The CellCelector is placed in the ALS Incubator-**Flow**Box with high CO2-atmosphere and heated environment (37°C), especially useful for long-term experiments and primary cell cultures.

Glossary

Propidium iodide (PI) –- is impermeant to cell membranes and generally excluded from living cells. It can be used for identifying dead cells in a population and as a counterstain in multicolor fluorescent techniques. Propidium iodide is an intercalating agent and a fluorescent molecule that can be used to stain DNA. When excited by 488 nm of laser light, it can be detected with 562-588 nm band pass filter. It can be useful for differentiation of necrotic, apoptotic and normal cells.

BrdU – Bromodeoxyuridine is a synthetic nucleoside analogue to Thymidine and can be used for tagging of proliferating cells in viable tissues. BrdU can be uptaken by the cell and gets integrated into the new DNA during S-phase instead of dTTP nucleotide. Antibodies can be used for immunochemical visualization of DNA containing integrated BrdU.

Nanog – transcription factor essential for self-renewal of hESC.

Oct-4 - transcription factor essential for primordial germ cell survival and maintenance of pluripotency.

SSEA-1 – Marker for differentiated cells

SSEA-1 antibodies detect a lactoseries oligosaccharide antigen expressed on the cell surface of mouse embryonal carcinoma and embryonic stem cells. The antigen is also found on early mouse embryos and both mouse and human germ cells. It is absent on human embryonic stem cells (hESC) and human embryonic carcinoma cells (hEC). Expression of SSEA1 in these human cell types increases upon differentiation, while on the mouse cell types differentiation leads to decreased expression.

hEC – human embryonal tetracarcinoma cells

Tra-1-60, Tra-1-80 – Tra-1-markers are antigens associated with the pericellular matrix proteoglycan upon the surface of hESC (also hEC, hEG, hES). The monoclonal antibodies Tra-1-60 and Tra-1-80 react with different epitopes on the same antigenic molecule.

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