

# Classification of single cell colonies into Holo-, Mero- and Paraclones using CELLAVISTA® and YT-SOFTWARE®

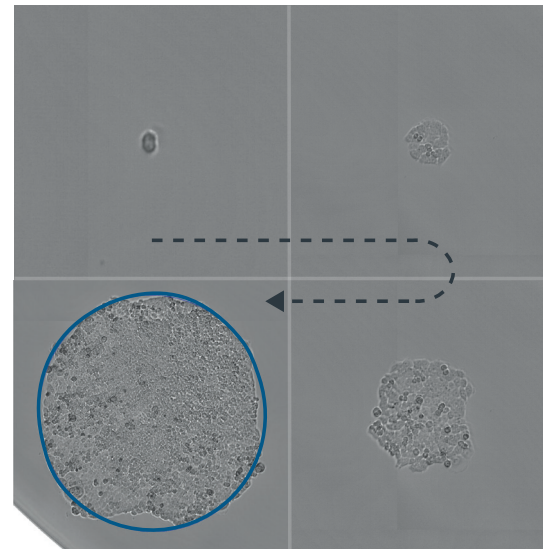
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## ABSTRACT

Stem cells are a powerful tool in fields like regenerative medicine due to their high proliferative and self-renewal capabilities. When seeded as single cells, they were attributed to form holoclones and differentiated, short-lived cells, which were termed meroclones and paraclones *in vitro*. These clones are distinguished by colony formation assays, in which the clones are fixed, stained and manually evaluated under a microscope. This assay does commonly not consider monoclonality, it is time-consuming, and it requires a lot of training and experience in order to discriminate the different colony types. Hence, to consider these shortcomings, we aimed to automate this assay. To do so, we performed single cell cloning experiments of pancreatic and colorectal cancer cells and automatically monitored colony growth over-time using CELLAVISTA®, YT-SOFTWARE® and our automation system. After 8-12 days, colonies were classified into three colony types by the image analysis application Single Cell Cloning (Holo, Mero, Para) of YT-SOFTWARE®. We verified the software-based classification by analyzing the expression of the proposed stem cell markers CD44 and Nestin in isolated holo- and paraclone cell lines by immunofluorescence stainings using NYONE® Scientific, CELLAVISTA® and the Virtual Cytoplasm (1F) application. The percentage of stained cells, as well as the average fluorescence intensity, of CD44 and Nestin were enhanced in holoclones compared to paraclones. Thus, the image analysis application Single Cell Cloning (Holo, Mero, Para) reliably distinguishes holoclones, meroclones and paraclones. Altogether, the procedure described in this note simplifies colony formation assays enormously and allows further cultivation of the detected clones making it a great tool for stem cell and cancer research as well as drug discovery.



KEYWORDS: SINGLE CELL CLONING, HOLOCLONE, MEROCLONE, PARACLONE, PROLIFERATION, STEM CELLS, STEMNESS, DIFFERENTIATION, CANCER RESEARCH, AUTOMATION, HIGH-THROUGHPUT, IMMUNOFLUORESCENCE STAINING

## SEED CELLS, START AUTOMATION, WALK AWAY, GET RESULTS

- Automation system conveniently images over time
- YT-SOFTWARE® proves monoclonality of the cells
- Image processing precisely detects and classifies colonies of different size and shape
- Method reduces hands-on time and allows high-throughput

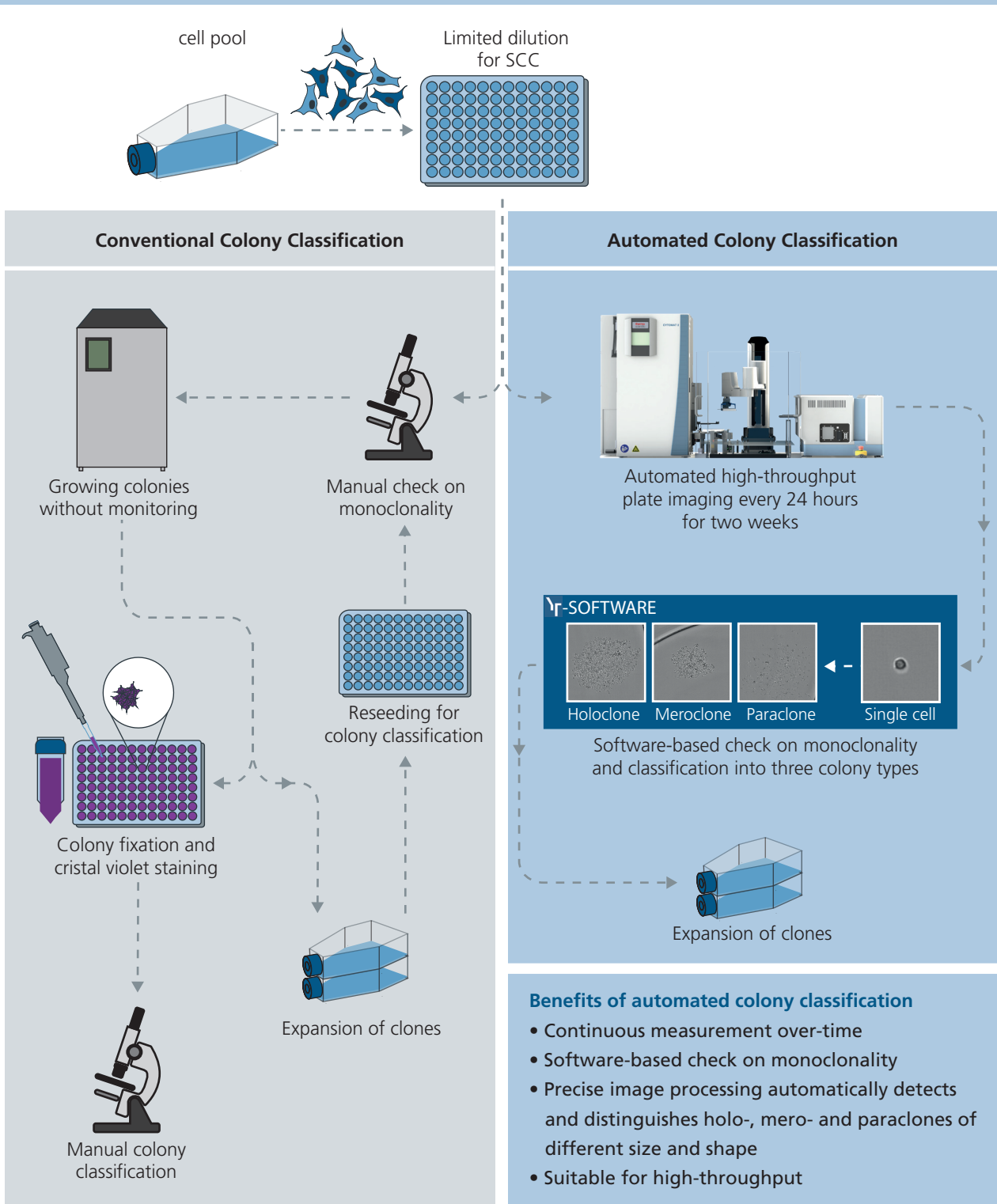


FIG. 1. OVERVIEW OF THE ASSAY PRINCIPLE IN COMPARISON TO CONVENTIONAL COLONY CLASSIFICATION

In order to obtain single cell colonies, 0.5 cells/well were seeded into a 96 well microplate. Left: schematic overview of a conventional colony classification assay including several time consuming steps. Right: SYNENTEC's automated colony classification assay. Cells were automatically imaged over-time using our automation system comprising CYTOMAT 2 C-LiN, SYBOT-1000®, CELLAVISTA® and YT-SOFTWARE®. Based on YT-SOFTWARE®, monoclonality was checked and once colonies had formed the **Single Cell Cloning (Holo, Mero, Para)** application was applied to automatically detect and classify colonies into holo-, mero- and paraclones. For further characterization, colonies can be expanded and analyzed *in vitro*.

## INTRODUCTION

Self renewal and repairment in the human epidermis is achieved by epidermal stem cells. Culturing these stem cells *in vitro* and diluting them to single cells generates single cell colonies known as holoclones, meroclones or paraclones [1]. Holoclones have an extensive proliferative potential and self-renewal capacity and generate meroclones and paraclones, which behave as transient amplifying progenitor cells that contribute to tissue heterogeneity. Conversion from holoclone to meroclone to paraclone is fulfilled through terminal differentiation [1]–[4]. The three colony types were distinguished based on their proliferative capacity, whereby holoclones have a high growth potential, while paraclones are not able to further proliferate and meroclones represent an intermediate type, forming growing and terminal colonies [1].

In addition to the discrimination of epidermal cell populations, the holo- mero-, paraclone classification was further extended to characterize cancer cells [5]. Similar to stem cells, cancer stem cells (CSCs) can proliferate indefinitely, have self-renewal capacity and divide asymmetrically giving rise to stem cells and differentiated, short-lived cells. These stem cells were attributed to be essential for tumor initiation, tumor progression, heterogeneity, metastasis, recurrence and therapy resistance [6]. The stem cell-like phenotype

of CSCs was associated with the morphology of holoclone colonies generated by single cells (figure 1). In addition to morphological differences, colonies also differ in their growth potential as well as in the expression of stem cell markers, and capacity to self-renewal, whereby these characteristics are most pronounced in holoclones.

The identification and classification of the three clonal types is challenging and time-consuming using conventional microscopes, making high-throughput experiments impossible. Moreover, the manual discrimination between clonal colony types by eye is based on subjective evaluations that limits reproducibility. Therefore, we aimed to develop an automated, efficient and accurate method to detect and classify single cell colonies into holo-, mero- and paraclones using our imaging device CELLAVISTA® combined with our automation system and the **Single Cell Cloning (Holo-, Mero, Para)** image analysis application of YT-SOFTWARE®. In order to validate the software-based discrimination between colony types, we generated clonal holo- and paraclone cell lines and analyzed the expression of stem cell markers by immunofluorescence stainings.

## MATERIAL

### Single Cell Cloning and Colony Formation

- Panc1 (adherent pancreatic carcinoma cells)
- HCT116 (adherent colorectal carcinoma cells)
- RPMI 1640 medium supplemented with 10 % (v/v) FCS, 1 % (v/v) L-Glutamine, 1 % (v/v) Sodium Pyruvate
- Trypsin 0.05 %/EDTA 0.02 % in PBS
- 96 well plates (e.g. CytoOne)
- SYNENTEC's imaging device (here CELLAVISTA®)
- SYBOT-1000® plate handler
- Cytomat™ 2 C-LiN incubator
- SYNENTEC's YT-SOFTWARE®
- Trypan Blue

### Immunofluorescence staining

- 96 well black glass plate (e.g. Greiner)
- Poly-D-Lysine, (Gibco, A38904-01)
- 4 % Paraformaldehyd in PBS
- 0.1 % (v/v) Triton X-100 in PBS
- 3 % (w/v) Bovine Serum Albumin (BSA)/0.1 % (v/v) Tween 20 in PBS
- PBS
- Hoechst 33342 (Invitrogen, H1399)
- Primary antibodies (Mouse anti-Nestin (10C2) antibody, Cell Signaling, Cat. No.: 33475; Mouse anti-CD44 (156-3C11) antibody, Cell Signaling, Cat. No.: 3570)
- Secondary antibody (Goat anti-mouse Alexa Fluor 488, Thermo Fisher Scientific, Cat. No.: A-11001)
- SYNENTEC's imaging device (here NYONE® Scientific and CELLAVISTA®)

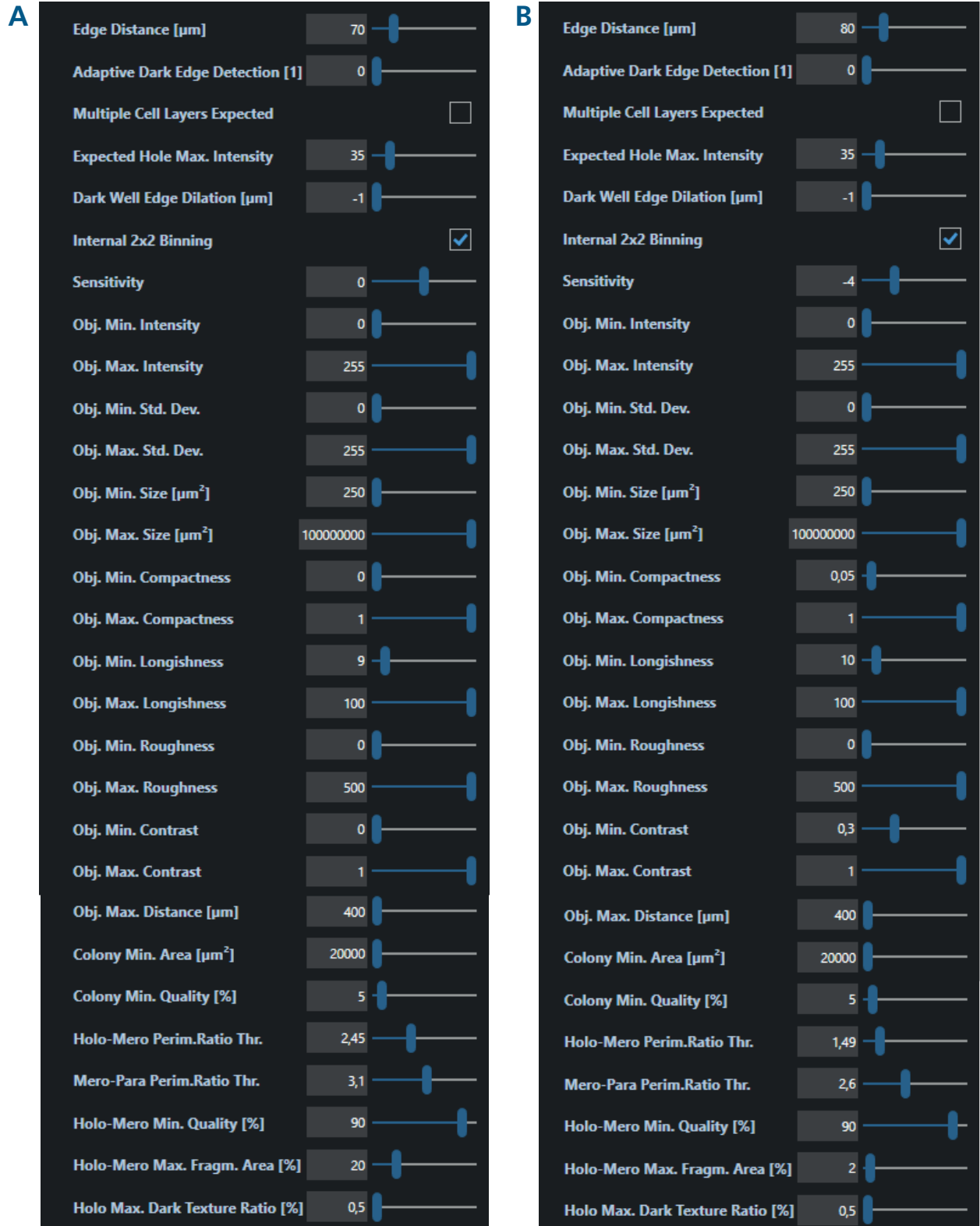


FIG. 2. IMAGE PROCESSING PARAMETER

Example of image processing settings of **Single Cell Cloning (Holo, Mero, Para)** application for (A) Panc1 and (B) HCT116 colonies.

## METHODS

### Cell culture and cell counting

The human pancreatic cancer cell line Panc1 and the human colorectal cancer cell line HCT116 were cultured in RPMI 1640 medium supplemented with 10 % (v/v) FCS, 1 mM GlutaMAX and 1 mM sodium pyruvate under standardized cell culture conditions (37 °C, 5 % CO<sub>2</sub>, humidified atmosphere). Cells were harvested at 70-80 % confluence with Trypsin and counted using SYNENTEC's **Trypan Blue** application. The required cell number was calculated referred to *viable cell density*.

### Single cell cloning and expansion of clonal colonies

For single cell cloning, the cell suspension was diluted to 2.5 cells/mL in medium and cells were seeded at a density of 0.5 cells/well (200 µL/well) into a 96 well plate using a multistep pipette. Afterwards, plates were centrifuged at 300 xg for 5 min to settle cells on the plate bottom and to remove air bubbles. Cells were imaged directly after seeding and then further every 24 h using CELLAVISTA®, SYNENTEC's automation system and the **Single Cell Cloning (Holo, Mero, Para)** image analysis application of YT-SOFTWARE®. To achieve optimal results during measurement, a 10x objective and the autofocus setting each image was chosen. Around 8-12 days after seeding, colonies were checked on monoclonality, and were classified into holo-, mero- and paraclones using the **Single Cell Cloning (Holo, Mero, Para)** application and the image processing parameters shown in figure 2. After 2 weeks, holo- and paraclones derived from a single cell

were transferred to 24 well plates and were further expanded in 6 well plates and cell culture flasks.

### Immunofluorescence staining of cells grown in microplate

Prior to cell seeding, 96 well glass plates were coated with 50 µL Poly-D-Lysine according to manufacturer's protocol. 5,000 cells/well (200 µL/well) were seeded in the coated wells using a multistep pipette. After 2 days, cells were fixed with cold 4 % paraformaldehyde/PBS (15 min). After washing three times with PBS (100 µL/well, 5 min) cells were permeabilized with 0.1 % Triton X-100/PBS (50 µL/well, 15 min), washed again twice (100 µL/well PBS, 5 min) and blocked with 3 % BSA/PBS and 0.1 % Tween 20 (50 µL/well) for 45 min at room temperature. Afterwards, cells were incubated with primary antibodies (here mouse anti-Nestin and mouse anti-CD44) diluted in blocking solution overnight (50 µL/well, 4 °C). Subsequently, cells were washed twice with PBS (100 µL/well, 5 min) and incubated with secondary fluorochrome-labelled antibody (here anti-mouse Alexa Fluor 488) diluted in blocking solution at room temperature in the dark (50 µL/well, 1 h). After two washes (100 µL/well PBS, 5 min), nuclei were stained by Hoechst 33342 diluted in PBS (50 µL/well, 10 min). Cells were washed twice (100 µL/well PBS, 5 min) and finally, 200 µL PBS were added to the wells. Immunofluorescence stainings were imaged and quantified using NYONE® Scientific (20x magnification) and CELLAVISTA® (40x magnification) and the **Virtual Cytoplasm 1F** application of YT-SOFTWARE®.

## RESULTS & DISCUSSION

### 1. Proof of single cell clonality and monitoring of colony growth

In order to generate and expand a clonal cell population, we seeded cells of the pancreatic cancer cell line Panc1 and the colon cancer cell line HCT116 at a density of 0.5 cells/well. The plates were automatically imaged directly after seeding and further every 24 h over two weeks using CELLAVISTA® combined with SYNENTEC's automation system and the **Single Cell Cloning (Holo, Mero, Para)** application of YT-SOFTWARE®. Once colonies had formed, they were automatically detected by the **Single Cell Cloning (Holo, Mero, Para)** application. The expansion from a single cell could be confirmed by going back to the first measurement. One feature of the image analysis application is the option to export a *Clone Gallery*. In this gallery, YT-SOFTWARE® automatically arranges every obtained picture per well with a time stamp in one figure, enabling a quick overview of growth area from single cell to colony (figure 3 A). In addition, YT-SOFTWARE® automatically evaluates and plots the colony area after image processing (figure 3 B). This gives an easy and quick overview of the proliferative capacity of every single cell clone. Exemplarily, the colony areas

of 5-8 Panc1 holo-, mero- and paraclones are shown in figure 3 C. On day 12 after seeding, the holoclones reached a median area of 1.08 mm<sup>2</sup>. In contrast, the paraclones grew to an area of 0.15 mm<sup>2</sup> and the meroclones showed a moderate growth of 0.42 mm<sup>2</sup> (figure 3 C). This is in line with published data describing holoclones as being the largest colony type [5].

### 2. Automated classification into three colony types

Once colonies had formed (Panc1 cells 12 days, HCT116 cells 8 days after seeding), the colony type was determined using the image analysis application **Single Cell Cloning (Holo, Mero, Para)** of YT-SOFTWARE®. The application automatically detects and analyzes the colony type, based on morphological characteristics. Holoclones are composed of small, densely packed, homogeneous cells with a clear and regular boundary. In contrast, paraclones are characterized by more differentiated, loosely packed, scattered and enlarged cells with irregular or fragmented boundary. Meroclones represent an intermediate type, with an irregular outline, consisting of a mixture of small densely packed cells and larger loosely packed cells [5]. These morphological characteristics lead

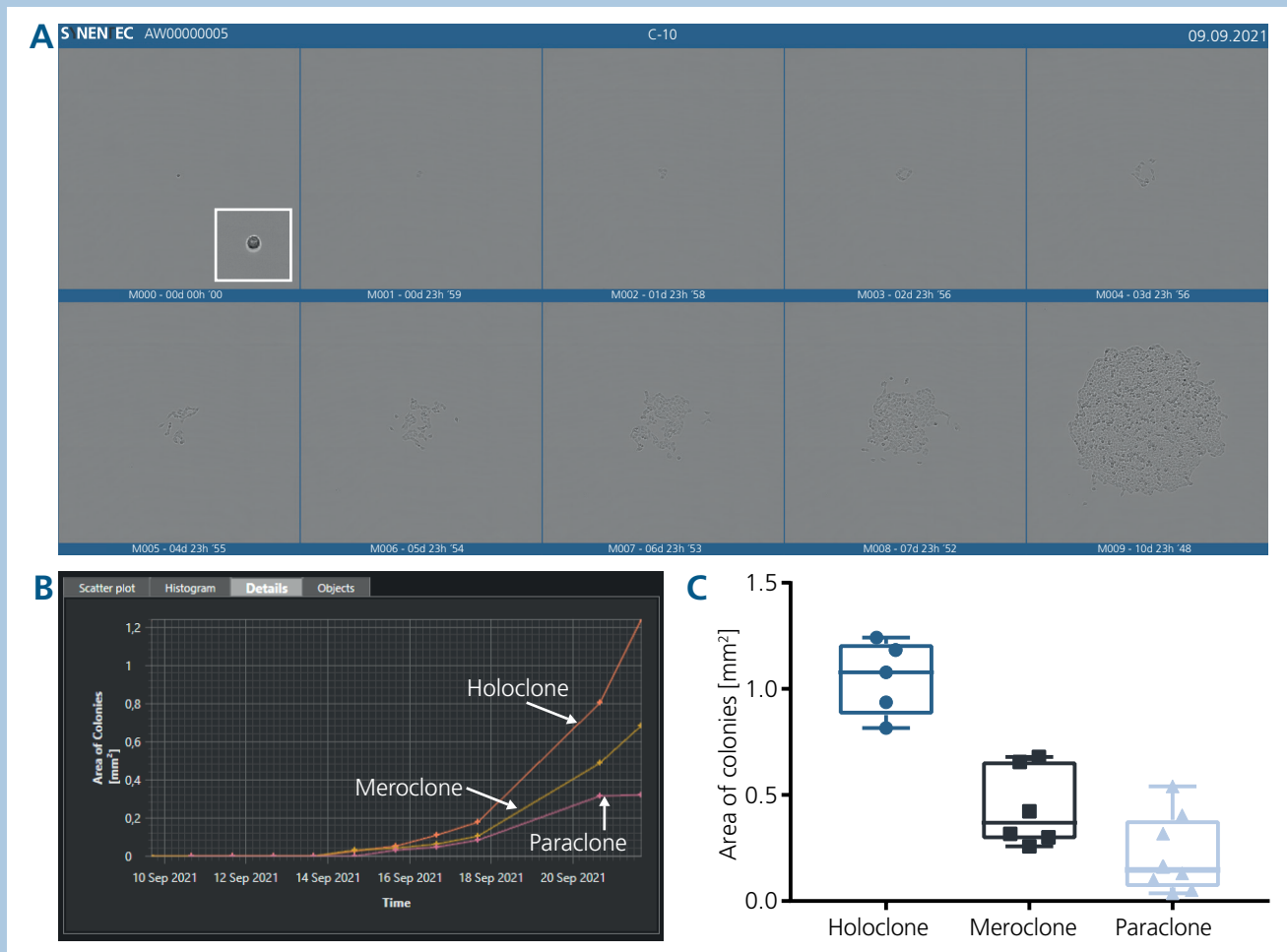


FIG. 3: GROWTH OF DIFFERENT COLONY TYPES

(A) Exported *Clone Gallery* of Panc1 holoclone with detailed information about the experiment, well and time created with YT-SOFTWARE®. (B) Representative *Area of Colonies* of holo-, mero- and paraclones shown in a time chart of YT-SOFTWARE®. (C) Colony area on day 12 after seeding of holo-, mero- and paraclones derived from single Panc1 cells. Data are presented as median and quartiles (Q1 as 25% and Q3 as 75%) of 5 to 8 colonies.

to four properties by which the software is able to discriminate the three clonal types: 1. the geometric shape, 2. the ratio of the clone area perimeter to the perimeter of an idealized ellipse, 3. the ratio of the loose clone area to the main area, 4. the ratio of particularly dark clone areas to total clone area. After image processing, the colony area is marked by a yellow border, while colony types are highlighted with an ellipse, colored blue for holoclone, green for meroclone and red for paraclone (figure 4 A,C). The proportion of holo-, mero- and paraclones gives hints about phenotypic characteristics of a cell line. Paraclones are supposed to be comprised of more differentiated cells, whereas holoclones are supposed to contain the highest proportion of CSCs. As previously described, Panc1 cells mostly formed paraclones (54.7 %), but they also gave rise to a considerable amount of mero- (22.5 %) and holoclones (22.8 %) (figure 4 B) [7]. In contrast, HCT116 cells mostly formed meroclones (74,3 %), less holoclones (18,9 %) and only a few paraclones (6,8 %) (figure 4 D).

### 3. Characterization of holo- and paraclones by immunofluorescence staining

In order to verify if the software-based categorization of the colony types gives reliable results, we analyzed Panc1 holo- and paraclones for the expression of stem cell markers CD44 and Nestin by immunofluorescence stainings. According to previous studies, holoclones are supposed to express higher levels of CD44 and Nestin in comparison to paraclones, since holoclones are supposed to contain the highest proportion of CSCs [7], [8].

For immunofluorescence stainings, holo- and paraclones derived from single cells were expanded to generate clonal holo- and paraclone cell lines. These cell lines were then reseeded at a density of 5,000 cells/well into 96 well black glass plates. After two days, cells were fixed and stained with mouse anti-Nestin or mouse anti-CD44 and anti-mouse Alexa Fluor 488 secondary antibody. Nuclei were visualized by Hoechst staining. Stainings were imaged with our automated imagers NYONE® Scientific (20x magnification) and

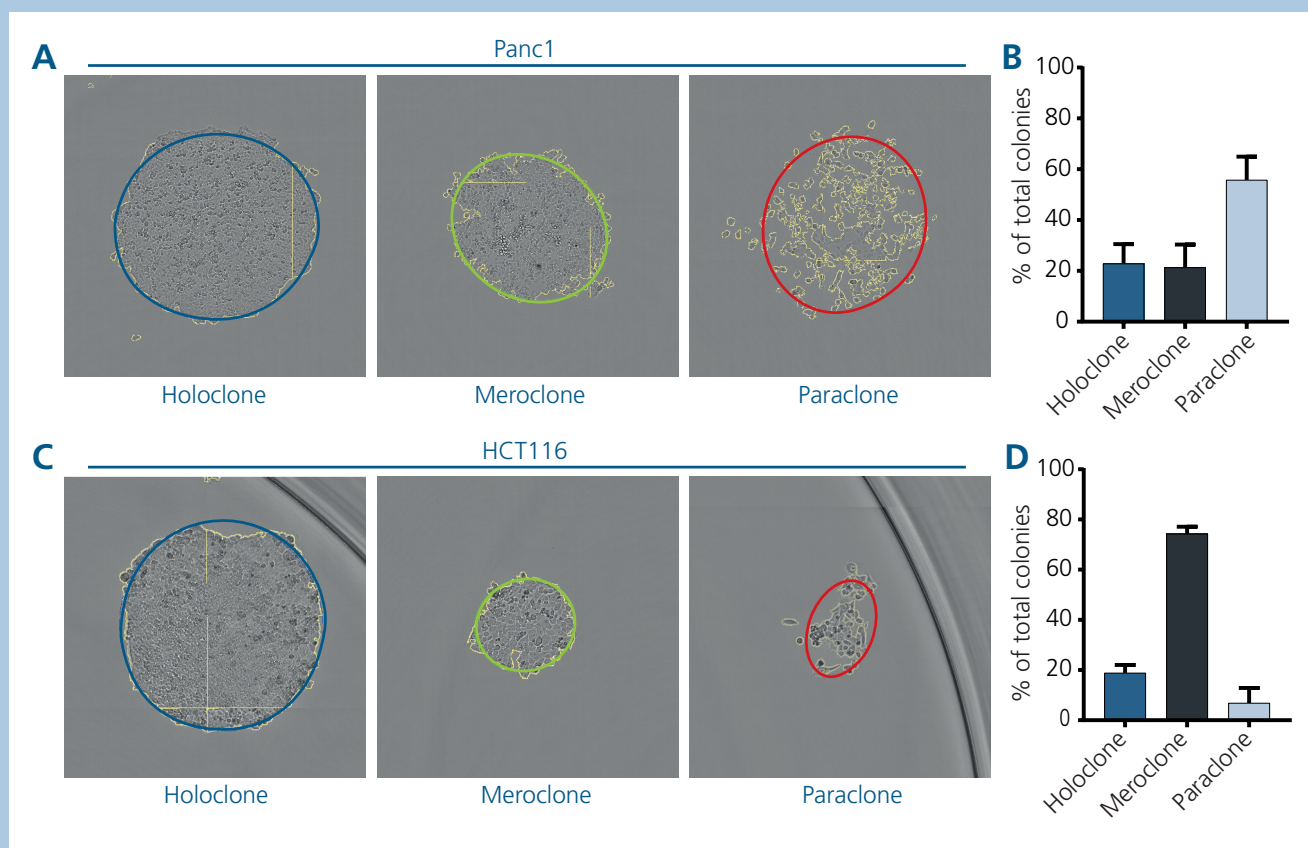


FIG. 4: CLASSIFICATION OF COLONIES INTO HOLO-, MERO- AND PARACLONES

(A, C) The image analysis application **Single Cell Cloning (Holo, Mero, Para)** of YT-SOFTWARE® automatically detects Panc1 (A) and HCT116 (C) colonies and marks the colony area with a yellow border. Colony types are highlighted by colored ellipses, blue: holoclone, green: meroclone and red: paraclone. (B, D) The percentages of different colony types of total number of colonies were determined for (B) Panc1 cells 12 days after seeding and (D) HCT116 cells 8 days after seeding. Data are presented as mean and standard deviation of 1-2 experiments, each performed in 2-5 technical replicates.

CELLAVISTA® (40x magnification) and the optical settings listed in table 1 (figure 5 A). Image analysis and quantification were performed using the **Virtual Cytoplasm 1F** application of YT-SOFTWARE® (figure 5 B, C). CD44 was expressed by both Panc1 holo- and paraclone, but the *percentage of stained cells* was slightly increased in holoclone (95.3 %) compared to paraclone cells (80.3 %) (figure 5 A, B). Moreover, also the *Average Fluorescence Intensity* was enhanced in holoclone (32.7) compared to paraclone cells (26.2) (figure 5 C). In contrast, Nestin was nearly exclusively expressed by holoclone cells indicated by 96.2 % stained cells, whereas only 13.8 % of paraclone cells were stained (figure 5 A, B). This was also reflected by an *Average Fluorescence Intensity* of 23.1 in holoclone and 3.9 in paraclone cells (figure 5 C). Summing up these results indicate that our **Single Cell Cloning (Holo, Mero, Para)** image analysis application reliably distinguished the colony types.

## CONCLUSION

Our automated imagers NYONE® Scientific and CELLAVISTA® combined with our automation system and YT-SOFTWARE® provide a valuable tool to detect, quantify and classify holo-, mero- and paraclones in a high-throughput manner. The image analysis application **Single Cell Cloning (Holo, Mero, Para)** enables a fast and precise generation of clonal cell lines. Colonies are automatically detected and classified into three colony types just by brightfield imaging. Therefore, they can further be cultivated as the detection requires no staining. Colony classification could be validated by checking the expression of markers proposed for CSCs by immunofluorescence stainings. Holoclones, which are supposed to contain the highest proportion of CSCs showed a higher expression of the stem cell markers CD44 and Nestin in comparison to paraclones, which are supposed to be comprised of more differentiated cells. Summing up, this application enables the automated and fast discrimination between stem cell-like cells from rather differentiated cells making it a great tool for research and drug discovery in the fields of stem cells, regenerative medicine, dermatology and cancer.

TAB. 1: IMAGING SETTINGS FOR NESTIN AND CD44 IMMUNOFLUORESCENCE STAININGS

Channel	Excitation Led [nm]	Emission Filter [nm]
Brightfield	Brightfield	Green (530/43)
Nestin/CD44	Blue (475/28)	Green (530/43)
Hoechst	UV (377/50)	Blue (452/45)

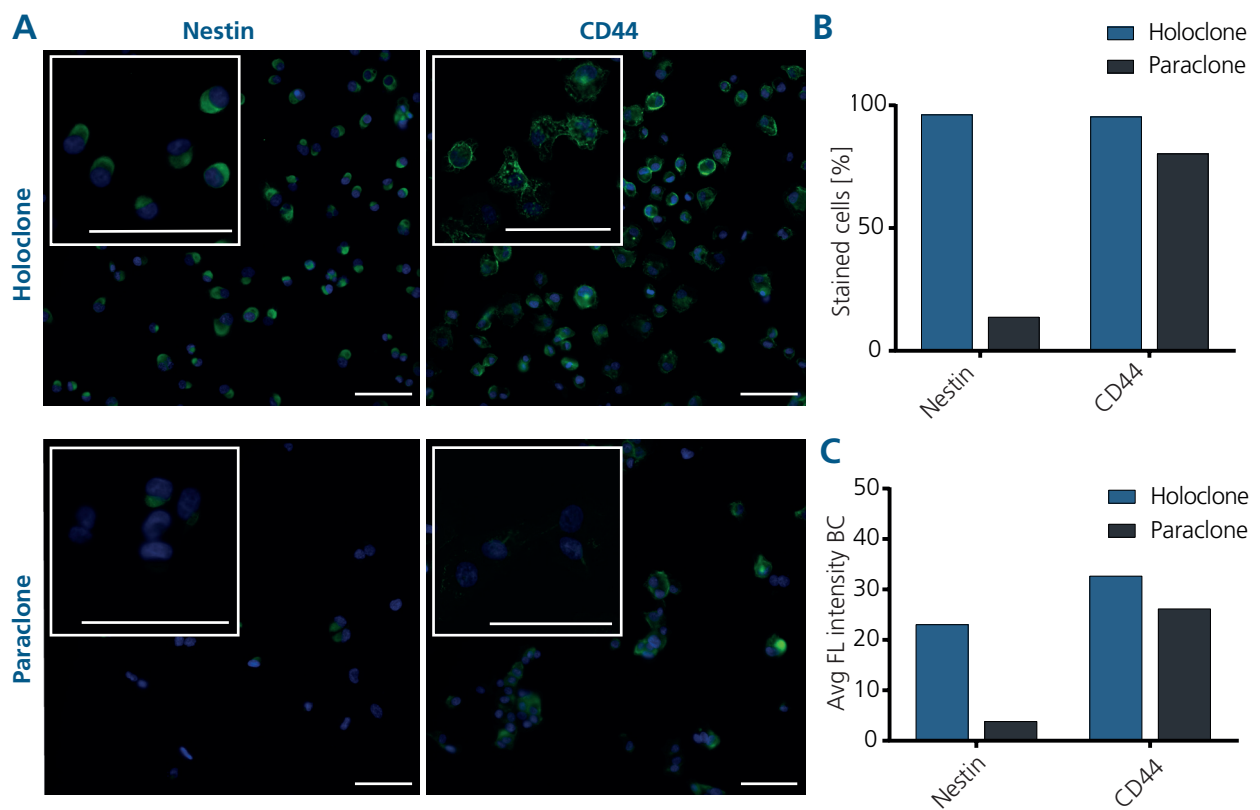


FIG. 5: EXPRESSION OF THE STEM CELL MARKERS NESTIN AND CD44 IN HOLO- AND PARACLONES

Panc1 holo- and paraclone cells (5,000 cells/well) were seeded in a 96 well microplate, fixed and stained using mouse-anti-Nestin or mouse-anti-CD44 antibody and anti-mouse Alexa Fluor 488 secondary antibody. Nuclei were visualized by Hoechst staining. Stainings were measured by NYONE® Scientific at 20x magnification and CELLAVISTA® at 40x magnification (white insert) and were evaluated with the image analysis application **Virtual Cytoplasm 1F** of YT-SOFTWARE®. Avg FL Intensity BC, Average Fluorescence Intensity Background Corrected; CD44, Cluster of Differentiation 44. (Scale bar: 100 µm)



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## Acknowledgement

We thank the Institute for Experimental Cancer Research, especially Prof. Susanne Sebens, for outstanding support, fruitful discussions and a great working atmosphere during this cooperation.



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published 07/2022

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