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VyCAP's Puncher Technology for Single Cell Identification, Isolation, and Analysis

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Abstract

Here we present the Puncher technology for the isolation of single cells. This technology combines a silicon chip with microwells, fluorescence imaging, and a punching method to isolate and transfer the single cells to standard reaction tubes. The technology is compatible with commercially available downstream workflows and instrumentation. Here we focus on the isolation of CTC but the Puncher technology can be applied to isolate single cells from liquid biopsies and more general from cell suspensions. It is especially suited for cell suspensions that contain:

- Cells of interest at a frequency of 1 per 10,000 or less
- A low total number of cells ranging from 1 to 100,000, that are present in a volume of 0.01 to 50 mL.

The frequency of appearance of CTC in blood is in the order of the 1 per 10^6 leukocytes. To be able to isolate the single CTC with the Puncher technology, enrichment of the CTC by a 3 logs reduction of the leukocytes is required. Here we describe the use of Rosettesep and Parsortix as examples of pre-enrichment methods that are compatible with the Puncher technology and further downstream applications. © 2018 International Society for Advancement of Cytometry

Key terms

single cell isolation; microwells; Puncher; circulating tumor cells; clonal expansion

Over the last couple of decades, multiple technologies have been developed for the enrichment and isolation of rare cells, including circulating tumor cell (CTC). Compatibility between these technologies, is however mostly not present (1–3). Enriched sample volumes are too large or the number of unwanted cells in the enriched samples is too high to identify and isolate the cells of interest. This leads to low recovery rates when isolating single CTC from the enriched samples. The Puncher technology for single cell isolation overcomes most of these issues.

Puncher Technology

The Puncher technology comprises of:

1. A disposable that contains a microwell chip to distribute single cells in individual microwells.
2. A fully automated Puncher system that comprises of
   - An imaging microscope that acquires high resolution images of single cells in the microwells.
   - A Punching part that isolates the cells of interest from the microwells.

Microwell Chip

The microwell chip contains 6,400 individual wells. The wells are present in an area of 8 × 8 mm, have a diameter of 70 μm and a depth of 360 μm. The resulting volume of a single microwell is 1.5 nL. The bottom of each well is closed with a

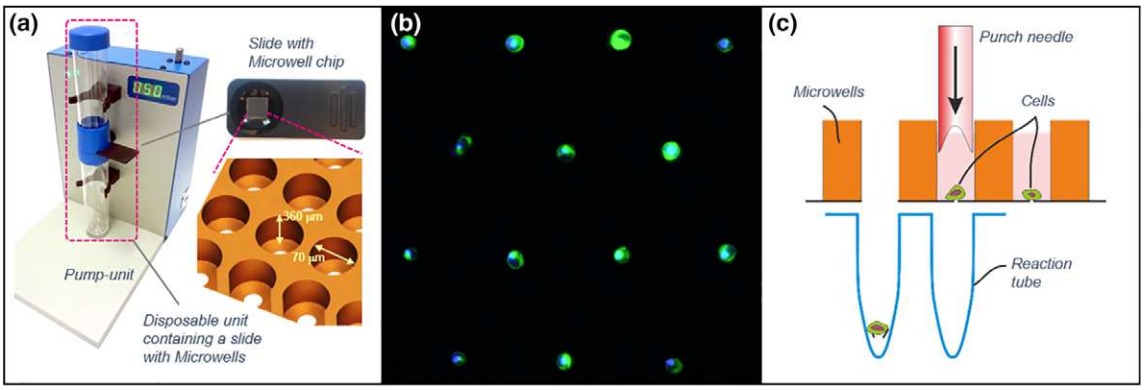

Figure 1. (a) Different parts used to distribute single cells into the microwell chip. The microwell chip is mounted in a slide that can be removed from the disposable. The pump-unit creates a small negative pressure below the microwells. (b) Fluorescence image acquired by the Puncher system of single MCF-7 cells distributed in individual wells. (c) Schematic representation of the isolation of single cells. A solid needle is lowered inside the well that contains the cell of interest and pushes the bottom plus cells from the microwells toward a reaction tube. [Color figure can be viewed at wileyonlinelibrary.com].

optically transparent silicon nitride (SiN) membrane with a thickness of 1 µm. This membrane is supplied with a single pore of a diameter that matches with the type of application. In case all objects present in the sample need to be collected the diameter of the pore is chosen to be small enough that none of the objects is able to pass through. For the isolation of CTC, a pore diameter of 5 µm is used.

Single Cell Distribution in the Microwell Chip

A cell suspension with a volume of 0.1–50 ml is transferred to the disposable. The microwell chip is mounted in a slide that is part of the disposable (Fig. 1A). The disposable is connected to a pump-unit that applies a negative pressure to the waste side of the disposable, below the microwells. This pressure pulls the sample fluid inside the microwell where it exists through the pore at the bottom. Fluidic forces drag the cells along the flow lines, into the wells and toward the pore (4). Once a cell has landed on the pore it blocks the flow into that well and no other cell will be pulled into the same well. The remainder of the sample will flow towards still available empty wells until the whole sample has passed through. Figure 1B presents an image of single cells captured inside the individual microwells. In case the sample contains a collection of cells with different diameters and flexibility, the pore diameter can be chosen such that the pore acts as a selection tool, collecting only the largest and least flexible cells.

Imaging and Identification of the Cells

To acquire images of the individual cells, the slide with the microwell chip is removed from the disposable and transferred to the Puncher system. The silicon nitride (SiN) bottom of the microwells is transparent and allows acquisition of high resolution fluorescence images. At maximum six fluorescence channels are available and these can be chosen by the user. Image analysis algorithms make a pre-selection of the cells that match the set criteria. Images of these cells are presented to the user who makes the final the selection of cells to be isolated and to which tube each cell needs to be transferred.

Isolation of the Cells

Isolation of the cells is achieved by punching out the SiN bottom of the microwell together with the cell that is present on the pore. This is done by lowering a solid needle into the microwell containing the selected cell. The alignment and shape of the needle is such that the tip of the needle does not touch the cell during punching. The punched bottom and cell both drop down into an Eppendorf tube, a PCR well plates or a culture plates, which is automatically positioned below the punched microwell. The current status of the technology isolates the cells at a rate of 1 cell/second with a single cell isolation efficiency of greater than 90%. After isolation, the DNA / RNA or protein content of the single cell can be analyzed. During the isolation process the cells stay alive and reside in buffer at all times.

Downstream DNA Analysis

Depending on the cell type, fixation and intended downstream analysis, different commercially available WGA reagent kits can be used. WGA of punched single cells using AMPLI-1 (# WG001R, Menarini, Florence, Italy) and Repli-G (Qiagen, Hilden, Germany) were successfully used in combination with the Puncher technology with minor modifications from the recommended WGA manufacturer's protocols (www.vycap.com/literature/protocols). The glass like SiN material of the microwell bottom that is isolated together with the cell doesn't interfere with the WGA reagents.

Most of the DNA analysis protocols require a number of PCR cycles. The Puncher technology is able to punch the cell directly into 0.2 mL Eppendorf PCR tubes to assure compatibility with the PCR instrumentation present in most laboratories. In general, the first step of WGA involves cell lysis. To limit the amount of WGA reagents and to ensure that the end volume of the WGA reaction is small enough to fit in a 0.2 mL Eppendorf tube, the lysing reagents have small

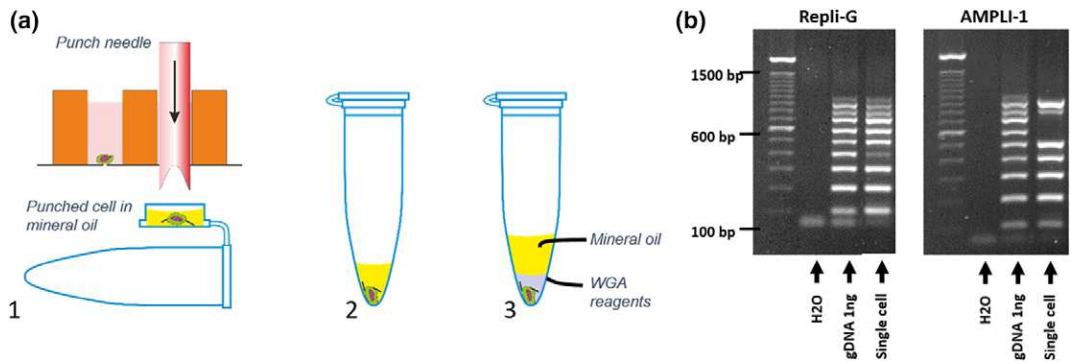

Figure 2. (a) Schematic presentation of punching a single cell and microwell bottom in mineral oil followed by addition of WGA lysing reagents. (b) Agarose gel images of WGA quality control kit for Repli-G and AMPL1. Lanes present a 100 bp ladder, H_2O, 1 ng of gDNA and the single cell WGA product of Repli-G and AMPLI1. [Color figure can be viewed at wileyonlinelibrary.com].

volumes of 1–5 μL. To make sure that the punched cell is actually inside this small volume of lysing reagent we have designed a methodology that contains three steps, which are schematically depicted in Figure 2A:

1. Punch the cell into the cap of an Eppendorf tube that is filled with 35 μL of mineral oil. This amount is sufficient to cover the whole surface of the cap.
2. Close the tube and spin down the oil containing the punched cell at 10,000*g*.
3. Add 1–5 μL WGA lysing reagent. Spin down the reagents at 10,000*g*. The punched cell will become submerged in the small reagent volume, and the WGA PCR cycle can be started.

In case of WGA reagents that use a lysis volume that is large enough to cover the bottom of the cap, the cell can be punched directly into the lysing reagent. For example, the recommended lysing reagent volume of the Repli-G WGA kit was increased to 8 μL, which is sufficient to make sure that the bottom is punched into the lysing reagent.

To determine the quality of the WGA product, a WGA quality control assay was developed. The assay targets 10 different DNA fragments, present on 10 different chromosomes using specific PCR primers. The presence of these fragments is determined using an agarose gel. Figure 2B presents two agarose gels showing the different 10 bands for Repli-G and 7 bands for AMPLI-1 (Menarini). The 3 bands missing in the AMPLI-1 single cell lane are due the presence of restriction sites of MseI (bands 606 and 720 bp) and the 1,009 bp band, which represents an AMPLI-1 3.4 kb fragment, being too large to be amplified by AMPLI-1.

Blood Acquisition

Anti-coagulants and preservatives used for blood collection greatly influence the results of isolation techniques that depend on the physical characteristics of the cells. For downstream CTC DNA applications no viable cells are needed and we optimized our protocols using Transfix blood collection tubes (#CTC-TVT-045, Cytomark, Buckingham, UK). These blood collection tubes allow VyCAP single CTC isolation for up to 48 h after blood draw and preserve cell morphology while the cells do still retain a certain flexibility that allows efficient separation of the excess leukocytes, while keeping the CTC in the pore at the bottom of the microwell. For applications in need of viable single cells, collection tubes without preservatives such as EDTA are required. Sample processing parameters depend on the type of preservative and time between the blood draw and processing. Protocols for CTC filtration and isolation are present at www.vycap.com/literature/protocols.

Results

Isolation of Single Cells

A SKBR-3 cell suspension with a volume of 1 mL was applied to the microwells and the single cells distributed across the individual microwells. Next the cells were identified and the bottoms of selected wells were punched from the microwells. Figure 3A presents several punched SKBR-3 cells together with the bottom of the microwells in which they resided. The cells were labeled with CellTracker Orange (C2927, Life technologies, Carlsbad) and Hoechst 33342 (H3570, Life Technologies). Figure 2B presents an image of a single MCF-7 cell that was punched from a microwell into a culture well plate filled with culture medium. The same cell was followed for several days and images of the proliferating cell were acquired at time point 0 h, day 2, 5, and 8 after the cell was punched from the microwell. The success rate of expanding Punched single cells to colonies is highly dependent on the type of cells. For MCF-7 cells a high success rate of 50%–70% can be obtained.

Isolation of Single CTC from Whole Blood

A blood volume of 7.5 mL from healthy donors was collected using Transfix blood collection tubes. A exact number of cells from the MCF-7 and SKBR-3 breast cancer cell lines and the PC3 prostate cancer cell line were spiked in the collected blood and left overnight at room temperature. The number of spiked cells ranged from 100 to 200 cells. The microwell chip contains 6,400 pores in as many microwells. The erythrocytes are flexible and pass through the pores easily. Leukocytes are more rigid and larger and will pass

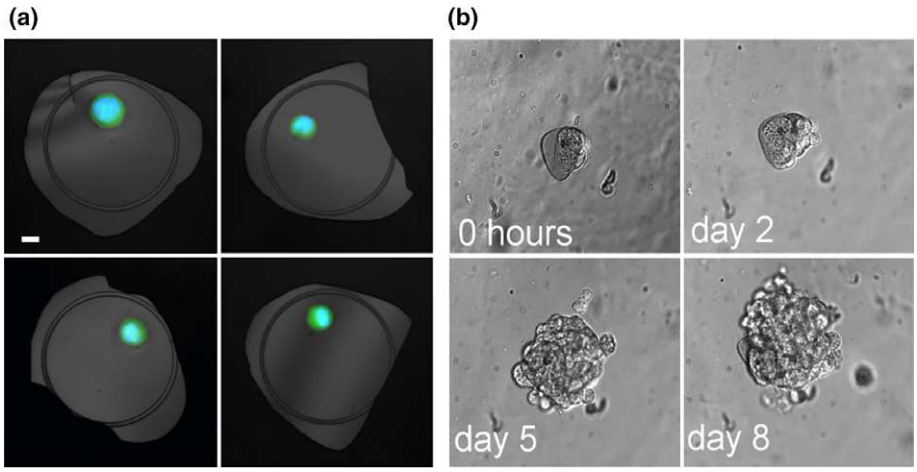

Figure 3. (a) Examples of punched bottoms with SBKR-3 cells. Cells are fluorescently labeled with CellTracker Orange and Hoechst 3334 and are located on the pore of the punched microwell bottom. (b) Clonal expansion of a single MCF-7 cell. [Color figure can be viewed at wileyonlinelibrary.com].

through the pore more slowly with the possibility of being captured on the pore. In order to isolate a single CTC from 7.5 mL of whole blood, it is required to reduce the leukocytes by 3 logs before transferring the sample to the microwell chip to avoid clogging of the pores. Of the leukocytes still present after depletion, most will pass through the 5 μm pore. The CTCs that are larger and more rigid than leukocytes will stay on the pore and will be distributed as single cell. CTC were identified as a nucleus containing cell that is Cytokeratin positive, and CD16 and CD45 negative (Cytokeratin CK11-PE, Cell Signaling Technology, #5075S; Cytokeratin AE1/AE3-eFluor 570, eBioscience, #41-9003-82; anti-CD45-APC, eBioscience, #17-0459-41; anti-CD16-APC, BioLegend, #302011; DNA, Hoechst33342, ThermoFisher, #H3570).

Enrichment by RosetteSep

RosetteSep is an immune-density procedure for the depletion of leukocytes from whole blood. By crosslinking the unwanted leukocytes to erythrocytes, the leukocytes can be separated from the target cells during standard density gradient centrifugation. Here we used the RosetteSep CTC Enrichment Cocktail Containing Anti-CD36 (#15127, StemCell Technologies, Vancouver, Canada). The used RosetteSep protocol was slightly modified from the recommended manufacturer's protocol (www.vycap.com/literature/protocols). After initial antibody incubation, the sample was centrifuged and the enriched fraction isolated using the SepMate tube (#85450, Stemcell Technologies). The final enriched sample volume is 50 μL. Next, fluorescent labels against cytokeratin, CD16 and CD45, and DNA were added to the 50 μL, according to protocol (www.vycap.com/literature/protocols). After completion of the labeling procedure the final sample volume of 500 μL was transferred to the microwells and the cells distributed across the individual microwells. Fluorescence images of the microwells were acquired and the tumor cells were identified. The number of collected tumor cells in the microwell was calculated as a percentage of spiked tumor cells.

The percentage of spiked cells that were collected in the microwells, after leukodepletion and distribution of cells across the microwells ranged from 40% to 50% (N = 5) for the PC3 and 5% to 15% (N = 5) for the MCF-7 cells. The lower recovery percentage for MCF-7 is mainly attributed to the presence of CD36 on MCF-7 and breast cancer cells in general.

Enrichment by Parsortix

The Parsortix system (Angle, Guildford, UK) uses a micro-fluidic technology in the form of a disposable cassette to capture and then harvest CTC from whole blood. The cassette captures CTC based on their less deformable nature and larger size as compared with other blood components. The Parsortix system was set-up according the manual using the cell separation cassette GEN3D6.5. Transfix collected whole blood with a volume of 7.5 mL, spiked with an exact number of either MCF-7 or SKBR-3 cells, was loaded onto the Parsortix system. After completion of the protocol for capturing and labeling, the collected cells were harvested from the cassette and an additional flush with 1 mL of PBS was performed, resulting in a sample volume of 1.2 mL. The enriched sample volume was directly transferred to the microwell chip where the cells distributed across the individual microwells. Fluorescent labels against cytokeratin, CD16 and CD45, and DNA were added to the microwells to label the cells inside the microwells. Fluorescence images of the microwells were acquired and the recovery was calculated as a percentage of spiked tumor cells. The overall recovery for the Parsortix enrichment in combination with microwell cell distribution and labeling, ranged from 35% to 65% (N = 5) for the SKBR-3 and 20% to 45% for the MCF-7 cells (N = 11). The lower recovery percentage for MCF-7 cell is attributed to the smaller size of the MCF-7 cells (5). This results in a lower possibility to collect these cells in the Parsortix cassette as well as a higher chance that these cells pass through the pores of the microwell chip.

Isolation by Punching

Isolation of the identified CTC is an efficient process with a success rate of greater than 90%, meaning that once a CTC is identified in the microwell, the chance that it is transferred successfully to the reaction tube/plate is greater than 90%. Previously we reported a success rate of 80% (4). Improved production methodologies of the microwells and punch needle improved this to over 90%. As such the percentage of cells that is collected in the microwells is almost equal to the percentage of cells that can be isolated into a reaction tube for downstream analysis or clonal expansion.

Conclusion

The Puncher technology uses a microwell chip to distribute single cells in 6,400 individual wells. Cell suspensions that contain a limited number of cells can be applied directly to the microwell chip to distribute single cells in individual wells. To process whole blood volumes of 7.5 mL pre-enrichment is needed. In principle any enrichment technology can be used (3). Here we described enrichment using RosetteSep and Parsortix in combination with the Puncher technology. These two enrichment methods have been chosen because of their commercial availability and validated protocols. Others have successfully used the Puncher technology in combination with the Cellsearch system (Menarini) (6). In case of pre-enrichment with the Parsortix system the sample volume after enrichment is 1.2 mL, with the Rosettesep method the enriched sample has a volume of 500 μL. These volumes can directly be transferred to the microwell chip without further concentration. Using these two technologies and starting with a whole blood volume of 7.5 mL, the enriched sample contains a number of leukocytes between 10 and 75 thousand. The pore in the bottom of the microwell serves as an additional separation on cell size and rigidity and removes most of the excess leukocytes. This allows processing of samples that contain a larger number of leukocytes than the available number of 6,400 microwells.

The RosetteSep CTC enrichment cocktail containing anti-CD36 resulted in the highest depletion of leukocytes in combination with a 40%–50% overall recovery. For breast cancer cells, StemCell recommends to use a different enrichment cocktail, for example the CD45 depletion cocktail or the CTC enrichment cocktail containing anti-CD56 (StemCell products #15122 and #15137). The authors have evaluated these kits but this resulted in lower leukocyte depletion and lower tumor cell recoveries as well as the formation of coagulates in the enriched sample. For breast cancer CTC we recommend to use a different enrichment technology and here we demonstrated the use of the Parsortix system. The Parsortix system is a size based method which resulted in a higher recovery for the larger SKBR-3 cells compared with the MCF-7 cells.

We presented workflows and the results for the isolation of single CTC from whole blood using the Puncher technology. The technology is well suited for the isolation of single cells from cell suspensions. Examples are the isolation of trophoblasts from maternal blood for non-invasive-prenatal testing (NIPT), isolation of single cells from cell suspensions obtained from for example the dissociated of Zebrafish embryos, organoids, or tissues or isolation of cells from cell lines and clonal expansion of single cells (7).

Ethics Statement

Blood samples were collected from healthy donors, and obtained from the TNW-ECTM-donor services (University of Twente, Enschede, the Netherlands). Blood collection was performed in accordance with the Dutch regulations and was approved by the Medical-ethical assessment committee Twente (METC Twente).

Conflict of Interest

Authors M. Stevens, L. Oomens, J. Broekmaat, J. Weersink, and A. Tibbe are employed by VyCAP. Authors J. Swennenhuis and F. Abali declare to have no potential conflict of interest.

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