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TITLE:

Micromanipulation of Circulating Tumor Cells for the Downstream Molecular Analysis and Metastatic Potential Assessment

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KEYWORDS:

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SHORT ABSTRACT (SUMMARY):

Here, we present an integrated workflow to identify phenotypic and molecular features that characterize circulating tumor cells (CTCs). We combine live immunostaining and robotic micromanipulation of single and clustered CTCs with single cell-based techniques for downstream analysis and assessment of metastasis-seeding ability.

LONG ABSTRACT:

Blood-borne metastasis accounts for the most cancer-related deaths and involves circulating tumor cells (CTCs) that are successful in establishing new tumors at distant sites. CTCs are found in the bloodstream of patients as single cells (single CTCs) or as multicellular aggregates (CTC clusters and CTC-white blood cell clusters), with the latter displaying a higher metastatic ability. Beyond enumeration, phenotypic and molecular analysis is extraordinarily important to dissect CTC biology and to identify actionable vulnerabilities. Here, we provide a detailed description of a workflow that includes CTC immunostaining and micromanipulation, ex vivo culture to assess the proliferative and survival capabilities of individual cells, and in vivo metastasis-formation assays. Additionally, we provide a protocol to achieve the dissociation of CTC clusters into individual cells and the investigation of intra-cluster heterogeneity. With these approaches, for

instance, we precisely quantify survival and proliferative potential of single CTCs and individual cells within CTC clusters, leading us to the observation that cells within clusters display better survival and proliferation in ex vivo cultures compared to single CTCs. Overall, our workflow offers a platform to dissect the characteristics of CTCs at the single cell level, aiming towards the identification of metastasis-relevant pathways and a better understanding of CTC biology.

INTRODUCTION:

The clinical manifestation of metastasis in distant organs represents the final stage of cancer progression and accounts for more than 90% of cancer-related deaths[1]. The transition from localized to metastatic disease is a multi-step process, often mediated by circulating tumor cells (CTCs)[2-4]. These cells are shed from the primary tumor into the blood circulation and are transported to distant organs, where they may extravasate and establish metastatic lesions[5,6]. Although solid tumors can release a relatively high number of CTCs, most CTCs are destined to die, owing to high shear forces in circulation, anoikis-mediated cell death, immune attack or limited capabilities to adapt to a foreign microenvironment[7]. Therefore, it is pivotal to establish tools that enable the dissection of the molecular features of those CTCs that are endowed with metastasis-seeding ability. Recent preclinical and clinical studies suggest that the presence and quantity of the single CTCs and CTC clusters is associated with a worse outcome in patients with various types of solid tumors[8-14]. CTC clusters are groups of two or more CTCs attached to each other during circulation and are more efficient in forming metastasis compared to single CTCs[3,15,16]. Cells within a cluster maintain strong cell-cell adhesion through desmosomes and adherens junctions, which may help to overcome anoikis[17,18]. Recently, we observed that clustering of CTCs is linked to hypomethylation of binding sites for stemness- and proliferation-associated transcription factors, leading to an increased ability to successfully initiate metastasis[19]. CTC cluster dissociation results in remodeling of key binding sites, and consequently, the suppression of metastatic potential[19]. Additional to clusters of cancer cells, CTCs can also associate to white blood cell (most frequently neutrophils) to maintain high proliferation levels in circulation and increase their metastatic capability[20]. However, many topics in the CTC field remain poorly understood, including the underlying molecular features and vulnerabilities of single and clustered cells.

In recent years, several strategies have been established that exploit cell-surface expression patterns as well as physical properties of CTCs for their isolation[21-25]. Antigen-dependent isolation methods rely mostly on the expression of cell surface Epithelial Cell Adhesion Molecule (EpCAM)[26]. The most frequently used and (at present) the only FDA-approved platform for CTC enumeration, is the CellSearch system, which is based on a two-step procedure to isolate CTCs[21]. In the first step, plasma components are removed by centrifugation, while CTCs are captured with magnetic ferrofluids coupled to anti-EpCAM antibodies. In the second step, the CTC-enriched solution is stained for nucleated (DAPI-positive) cells expressing cytokeratin (CK)[8,18,19], while white blood cells (WBCs) are identified using the pan-leukocyte marker CD45. Finally, captured cells are placed on an integrated screening platform and CTCs are identified through the expression of EpCAM, CKs, and DAPI while being negative for CD45. Although this is considered to be the gold standard for CTC enumeration, downstream molecular analysis is challenging with this technology due to inherent constraints in CTC retrieval. Additionally, given

its isolation procedure, it may favor the enrichment of CTCs with higher EpCAM levels compared to CTCs with lower EpCAM expression, due for instance to cancer heterogeneity[27] or downregulation of epithelial markers[28,29]. To overcome these limitations, antigen-independent technologies for the enrichment of CTCs have emerged. For example, the CTC-iChip integrates hydrodynamic separation of nucleated cells, including CTCs and WBCs from remaining blood components, followed by an immunomagnetic depletion of antibody-tagged WBCs, allowing purification of untagged and viable CTCs in solution[25]. Additionally, the fact that most CTCs are slightly bigger than red blood cells (RBCs) or WBCs led to the development of size-based CTC enrichment technologies[23,30] (e.g., the Parsortix system (ANGLE)) which makes use of a microfluidic-based technology, comprising a narrowing channel across the separation cassette, leading cells to a terminal gap of either 10, 8, 6.5 or 4.5 µm (different sizes are available depending upon the expected diameter of target cancer cells). Most of the blood cells pass through the narrow gap, while CTCs get trapped due to their size (but also due to their lower deformability) and are, therefore, retained in the cassette. Reverting the flow direction enables the release of captured CTCs, which are in a viable state and suitable for downstream analysis. Independently of the chosen protocol for CTC isolation, however, typical post-enrichment procedures still yield CTCs that are mixed with a relatively small number of RBCs and WBCs, making the analysis of pure single or bulk CTCs challenging. To address this issue, we established a workflow that allows CTC manipulation without potential bias introduced by blood cell contaminants. The addition of immunostaining beforehand, with variable antibody-combinations, distinguishes CTCs from blood cells and even allows to identify CTC subgroups with distinct surface-marker expression profiles. This highly customizable procedure can be then further combined with specific downstream applications.

Here, we describe a workflow that starts from a CTC-enriched product (obtained with any CTC enrichment technology of choice) and combines several approaches to gain insight into CTC biology at single-cell resolution. In a nutshell, our workflow enables the identification of single CTCs and CTC clusters by live immunostaining, followed by single-cell micromanipulation and downstream analysis using ex vivo culturing protocols, single cell sequencing, and in vivo metastasis assays.

PROTOCOL:

All the procedures involving blood samples from patients were performed upon signed informed consent of the participants. Procedures were run according to protocols EKNZ BASEC 2016-00067 and EK 321/10, approved by the ethical and institutional review board (Ethics Committee northwest/central Switzerland [EKNZ]), and in compliance with the Declaration of Helsinki.

All the procedures concerning animals were performed in compliance with institutional and cantonal guidelines (approved mouse protocol #2781, Cantonal Veterinary Office of Basel-City).

1. Patient sample preparation

1.1. Before starting, ensure to work with aseptic solutions and materials to maintain sterility

during the entire procedure.

1.2. Withdraw 7.5 mL of peripheral blood from a breast cancer patient into a 10 mL EDTA blood collection tube.

1.3. Incubate the Blood-containing tubes for a short time period (up to 1 h) at room temperature (RT) on a rocking shaker at 40 oscillation per minute (osc/min).

1.4. Enrich for single CTCs and CTC clusters using a CTC isolation method of choice[21-23,25]. Release CTCs in a 1x DPBS solution.

NOTE**:** Depending on the chosen CTC-enrichment technology, remaining white blood cells and red blood cells, might be present. Adjust the releasing pressure in order to preserve the CTC cluster structures.

1.5. Proceed immediately to the next step in section 3.

2. Mouse sample preparation

2.1. Before starting, prepare a 1 mL insulin syringe with a 25G needle, 5 mM EDTA filtered solution, and 2 mL EDTA blood collection tubes.

2.2. Ensure to work with aseptic solutions and materials to maintain the sterility during the entire procedure.

2.3. Pre-wash the syringe with 5 mM EDTA solution.

2.4. Load the syringe with 100 μL of 5 mM EDTA and remove bubbles.

2.5. Euthanize the mouse using 80% CO_2 / 20% O_2 gas inhalation and proceed immediately to the next step.

NOTE: An alternative to CO_2 inhalation method is the isoflurane anesthesia (3 vol% isoflurane and oxygen (carrier gas) at the flow rate of 600 mL/min) followed by cervical dislocation, or overdosing injection of ketamine/xylazine solution. Specific euthanasia method may vary depending on approved mouse protocol.

2.6. Confirm the animal's death by the absence of breathing activity, missing corneal reflex, and urination without external stimuli.

2.7. Perform a cardiac puncture by carefully introducing the needle of the EDTA pre-loaded syringe with a 30° angle into the thorax from the sternum towards the heart.

NOTE: For inexperienced experimenters, it is recommended to open the chest cavity first, before

performing the cardiac puncture, to better visualize the heart.

2.8. Retrieve up to 1 mL of blood. Without releasing the plunger, pull out the syringe from the animal chest, safely remove the needle, open the lid of the 2 mL EDTA blood collection tube and dispense the blood directly inside. Close the lid and invert the tube 10x.

2.9. Incubate the blood-containing tubes for a short time period (up to 1 h) at RT on a rocking shaker at 40 osc/min.

CAUTION: The needle must be disposed of in sharp-safe biological hazard disposal.

NOTE: Multiple punctures are possible to increase the total blood volume. Use separate syringes pre-loaded with EDTA for different withdraws to avoid aspirating clotted blood present in the previous needle.

2.10. Enrich for single CTCs and CTC clusters using CTCs isolation method of choice[21-23,25]. Release CTCs in a 1x DPBS solution.

NOTE: Depending on the chosen CTC-enrichment technology, remaining white blood cells and red blood cells, might be present. Adjust the releasing pressure in order to preserve the CTC cluster structures.

2.11. Proceed immediately to the next step in section 3.

NOTE: For all the following procedures, ensure that unfixed and freshly-isolated blood is used.

3. CTCs live immunostaining

3.1. Centrifuge CTC-enriched cell suspension at 72 x *g* for 4 min.

NOTE: 72 x *g* corresponds to 800 rpm if the rotor has a diameter of 100 mm. Convert the g-force number according to the rotor.

3.2. Gently resuspend the pellet in 1% BSA solution in 1x DPBS and add 2 μg/mL of anti-human EpCAM-AF488 antibody and 1 μg/mL of anti-human CD45-BV605 antibody or 2 μg/mL anti-mouse CD45-BV605 antibody in a total volume of 500 μL.

NOTE: For breast cancer patient-derived CTCs, anti-human EGFR-FITC, and anti-human HER2-AF488 can be added additionally to anti-EpCAM and anti-CD45. This allows to better identify the CTCs that are expressing lower EpCAM levels.

3.3. Incubate for 30 min at RT and protect from light.

3.4. Wash CTCs suspension using 1 mL of 1% BSA in 1x DPBS solution and centrifugation at 72 x

g for 4 min. Repeat this wash twice.

3.5. Resuspend stained cells in 2 mL of 1% BSA in 1x DPBS solution and transfer the total volume in 1 well of a 6-wells ultra-low attachment plate.

3.6. Incubate the plate for 10-15 min at 4 °C protected from light to allow partial sedimentation of the CTCs and residual blood cells.

4. Micromanipulation of CTCs and single-cell picking

NOTE: Before starting, be aware that the micromanipulator requires up to 45 min for complete set up. Once set up, the procedure for CTC identification and micromanipulation requires up to 2 minutes per cell (or cluster).

4.1. Make sure to terminate the CTC picking procedure within 2 h from the end of the staining.

4.2. Start up the micromanipulator software and switch on the micromanipulator. Connect the micromanipulator to the computer and initialize the robotic arm as well as the microscope stage by pressing the **Connect** button followed by **Int device**.

4.3. Close the protecting cabinet after every manipulation of the machine to be able to maneuver it through the computer.

4.4. Clean the surfaces from dust and spills by spraying ethanol and wiping the internal surfaces of the cabinet. A clean environment will ensure the sterility of the process.

4.5. Install a new glass capillary on the robotic arm. For the single-cell picking, use 20-30 μm capillary while 30-50 μm is preferable for CTC cluster picking.

4.6. Remove all bubbles present in the tubing by dispensing or aspirating the system oil.

CAUTION: Glass capillary is sharp and fragile. Handle with caution.

4.7. Fill the sterilization tank 1 with 70% ethanol, the sterilization tank 2 with sterile nuclease-free H_2O and the buffer tank with sterile 1x DPBS.

CAUTION: Do not close the lids of the tanks, since during the next steps the capillary will need to freely access the tanks.

4.8. Sterilize the capillary twice with 70% ethanol.

4.9. Replace the sterilization tank 1 with tank 2 and wash capillary in H_2O at least three times using the sterilization function.

4.10. Using the micromanipulator software, start a new experiment and choose the type of picking experiment between the automatic and the manual selection modes.

NOTE: Choose the type of experiment based on the end point of the manipulation. Manual mode enables maneuvering of a robotic arm after the capillary enters the cell suspension. This step is necessary for dissociation of CTC clusters into individual cells. It is possible to change the type of picking during the experiment.

4.11. Configure the deck tray specifying the position of the sterilization tank (liquid 1), buffer tank (liquid 2) and depositing tray (target 1 or 2). Target 1 allows to deposit into plates, target 2 allows to deposit into PCR tubes or PCR plates.

4.12. Set the temperature of the liquid tanks and depositing tray chosen to 4 °C.

NOTE: The picking process can be time consuming. Cooling of the liquid tanks and depositing tray is therefore advised

4.13. Position the ultra-low attachment plate containing the released CTCs solution under the microscope inside the micromanipulator cabinet. Remove the lid from the plate and close the cabinet. Keep the plate at RT for the rest of the set up and during the picking procedure.

4.14. Manually select the microscope objective for picking (10-20x) and the exposure time of all the necessary channels (Brightfield, FITC, and TRITC channels).

4.15. Select **show well navigator** from the toolbar to visualize and select the type of pickup plate (6-well plate).

NOTE: Any plate or well format can be installed by the manufacturer only.

4.16. Calibrate pickup position in the middle of the well containing the CTCs solution, but with no cells in the center of the field of view. Calibration performed at the edges of the wells can result in faulty calibration due to the uneven well surface.

4.17. Use the sensor to gently touch the bottom of the plate with the capillary (speed 0.01 mm/step and 1% speed).

4.18. Set the pickup position 0.05 mm above the bottom of the plate.

NOTE: Make sure to open and remove lids of plates and tanks before proceeding. The capillary may break on a closed or unremoved lid.

CAUTION: If the capillary breaks upon contact with lids, broken glass may be found in the surroundings or in the solutions.

4.19. Select cell type and picking parameters. Picking parameter can be set-up and loaded at each startup. For single-cell picking, select the manual mode.

4.20. Within the **Picking preparation** settings:

4.20.1. Set the **airgap volume** between the system oil and the sample to 1 µL

4.20.2. Set the **buffer liquid volume to take up** before each picking to 0.5 µL.

NOTE: Buffer liquid volume is adjusted to the total maximum pickup volume. Small volumes do not affect the picking or depositing efficiency.

4.20.3. Set the **speed of aspiration** of the buffer liquid between 1-6%.

4.20.4. Set the **waiting time** after aspiration of the buffer to 1 s.

NOTE: The option of taking up the buffer from target well instead of buffer liquid tank can be used if necessary

4.20.5. Set to **reuse glass capillaries** and **no sterilization between picks**.

NOTE: Sterilization between picks can be performed manually if required. Perform multiple washes in H_2O between picks or two sterilization in ethanol followed by multiple washes in H_2O.

4.21. Within the **Picking** settings:

4.21.1. Change the camera settings while picking and exposure time under the microscope to 7500 µs.

4.21.2. Select **fixed picking height**.

NOTE: Tool sensor or autofocus could be chosen instead. However, small imperfections of the plate can disturb the sensitivity of the sensor or the autofocus which may cause an impact of the capillary into the plate.

4.21.3. Set **aspiration speed of the capillary** entering the source plate to 7-15%.

4.21.4. Enable the **interactive picking**.

4.21.5. Set the **aspiration volume** in µL to 0-0.05.

4.21.6. Set the **speed** of aspiration to 5%.

4.21.7. Set the **waiting time** after aspiration in s to 0-1.

4.21.8. Set **the number of picked particles** per capillary to 1.

4.21.9. Set **no multiple picking at the same position** and **no scraping** functions. Aspiration properties must be set according to the type of experiment (e.g., automatic picking mode may require bigger aspiration volumes).

4.22. Within the **Deposit** settings:

NOTE: Depositing settings strictly depend on the depositing plate/tube.

4.22.1. For single-cell picking, define the **speed of capillary** entering the target plate to 25%.

4.22.2. Set the **speed** of dispensing to 6%.

4.22.3. Set the **waiting time** after dispensing in s to 0.

4.22.4. Set the **amount of airgap** dispensed in the target well to 100%.

4.22.5. Set **no rinse after depositing**.

NOTE: Sterilization can be performed after depositing to clean the capillary.

4.22.6. Set **maximum amount of particles deposit** per well and the number of targets to distribute particles to 1.

4.22.7. Calibrate deposit height on position A1 of target 1 for plates or on position A1 of target 2 for tubes. Place an open tube or plate without lid for the calibration and use the sensor to gently touch the depositing well (speed 0.01 mm/step and 1% speed). Set the **depositing height** 1 mm above the bottom of the plate.

4.23. Within the **Settings**:

4.23.1. Set the **stage speed** during picking to 5-10%.

NOTE: Fast movements of the plate may result in movement of cells in suspension and difficulties in multiple cell picking due to mispositioning.

4.23.2. Set **sterilization properties** using rinsing volume to 1 µL, 3 rinsing loops and 2 s of waiting time per sterilization round.

4.23.3. Apply the changes before closing.

4.24. Navigate the glass capillary using the joystick in the well and place it on top of the single

cell of interest. Choose picking up positions at a safe distance from the well border (1 mm) as the capillary may break on the edge of the well.

4.25. Manually add particles using the button of the joystick or manually selecting from the menu.

4.26. Select **pick activated particles** to start the picking.

4.27. The capillary will stop 0.05 mm above the bottom of the plate and on top of the selected pickup position because of the interactive picking (manual mode). Gently turn the knob of the joystick clockwise to manually aspirate the particle and the surrounding DPBS solution. The maximum volume is not fixed when the manual mode is on. However, the maximum volume allowed in the syringe will be 25 μL.

4.28. Dispense the excess DPBS solution or unwanted particles by gently turning the knob of the joystick counterclockwise. Too much dispensing will release the airgap of the syringe forming bubbles in your solution and compromising visibility.

4.29. Press **next** to proceed with the deposit.

4.30. Observe the capillary entering the depositing PCR tube or PCR plate, releasing the volume and going back to starting position with an empty capillary.

NOTE: If there is volume left in the capillary, proceed with sterilization. Then, re-check the settings, oil level, and oil height before performing a new picking.

4.31. Proceed from point 26 of section 4 to start a new single-cell picking. During picking it might be necessary to replace the capillary. After the installation, a new calibration of the pickup position must be performed. At the end of the calibration, select the function **Apply calibrated changes in Z-height to deposit height** in order to correct the deposit height to the new capillary calibration and to skip the deposit height calibration (section 4.16).

NOTE: The steps described in section 1-4 apply to most of the CTCs enrichment and isolation methods. Here we report optional steps for specific CTCs analysis.

5. Single-cell picking and seeding for survival and proliferation analysis

5.1. Ensure to work with aseptic solutions and materials to maintain sterility during the entire procedure.

5.2. Prepare a 384 well plate to contain the picked single CTCs for culturing with 20 μL CTC culturing media[31]. Ensure that CTCs are seeded in small volumes of the medium after manipulation (e.g., 10-20 μL for a 384 well plate).

5.3. Spin down the solution to the bottom of the plate. Place 384 well plate into the target 1 and keep at 4 °C.

5.4. Perform all the steps with the micromanipulator described in the section 4 to set up the micromanipulator and start a new picking.

NOTE: Multiple selections of the single cells will cause the formation of a picking list. The particles will be picked and deposited one by one in consecutive wells (see section 4.22.6). However, the interactive mode (manual mode) will stop the capillary right before the aspiration, therefore allowing the experimenter to control this critical step and to ensure successful picking. Fast movements of the plate may result in the movement of the cells in suspension and difficulties in multiple cell picking.

5.5. After depositing, repeat step 4 to start a new picking.

5.6. At the end of the picking, centrifuge the plate at 72 x *g* for 4 min to ensure that the picked cells are placed at the bottom of the well.

6. CTC cluster breaking and single-cell picking for sequencing

6.1. Ensure to work with aseptic solutions and materials to maintain the sterility during the whole procedure.

6.2. Prepare single PCR tubes or a PCR plate that will contain the single cells of the broken cluster with the total 2.5 µL cell lysing solution comprising 1 U/µL of RNA inhibitor.

6.3. Quickly spin down the solution to the bottom of the tube. Place the depositing containers into the target 2 and keep at 4 °C.

6.4. Perform all the steps described in section 4 with the micromanipulator to set up the micromanipulator.

6.5. Start a new picking. The capillary will stop 0.05 mm above the bottom of the plate and on top of the selected CTC cluster because of the interactive picking (manual mode).

6.6. Gently turn the knob of the joystick clockwise to manually aspirate the cluster and the surrounding DPBS solution. Dispense the aspirated volume including CTC cluster by gently turning the knob of the joystick counterclockwise.

6.7. Repeat the aspiration/disposal of the CTC cluster described in step 6 until the single cells forming the cluster will break apart.

NOTE: Too much dispensing will release the airgap of the syringe forming bubbles in your solution and compromising visibility.

6.8. Follow by eye the position of the single cells. Manually add the particles using the button of the joystick or manually selecting from the menu.

NOTE: Multiple selections of the single cells will cause the formation of a picking list. The particles will be picked and deposited one by one in consecutive PCR tubes/wells (see section 4.22.6). However, the interactive mode (manual mode) will stop the capillary right before the aspiration, therefore, allowing the experimenter to control this critical step and to ensure successful picking.

6.9. Select **pick activated particles** to start the picking.

NOTE: The amount of cell lysing solution will allow a single cell to completely lyse. However, long exposure of the lysed content to the lysing agent may degrade DNA or mRNA. Therefore, proceed immediately to the next step.

6.10. Immediately close the tube containing the picked single cell and transfer on dry ice for snap freezing.

6.11. Quickly spin the tube and check for the absence of drops on the sides of the tube to ensure a proper lyse of the deposited cell.

6.12. Repeat from step 5 to start a new picking.

NOTE: Microscope stage will move from one added particle to another at the speed described in section 4.23.1. CTCs suspension in the ultra-low attachment plate may move, causing faulty picking due to mispositioning.

7. CTCs isolation for mouse injection

7.1. Ensure to work with aseptic solutions and materials to maintain sterility during the entire procedure.

7.2. Prepare a PCR tube with 5 μL of sterile 1x DPBS.

7.3. Quickly spin down the solution to the bottom of the tube. Place the depositing containers into the target 2 and keep at 4 °C.

7.4. Within the **deposit** settings apply a change at the step 4.22.6: Set the **maximum amount of particles deposit per well** to 1000 and the **number of targets to distribute particle**s to 1. This step ensures that the picked cells will be deposited in the same tube for 1000 times.

7.5. Use only the interactive mode (manual mode) to precisely control the picking step and keep the picked solution free from contaminant cells.

NOTE: If more than 1000 cells are needed, increase the **number of targets to distribute particles** to avoid loss of sample after 1000 cells.

7.6. Continue the steps of section 4.26 to start a new picking. Repeat step 6 to perform multiple pickings. Annotate the number of cells collected at each picking in order to keep track of the number of cells available for the mouse injection.

7.7. At the end of the picking, centrifuge the tube at 72 x *g* for 4 min (see section 3.1.) and aspirate supernatant.

7.8. Resuspend the collected CTCs into the buffer of choice, suitable for mouse injection. For mammary fat pad injection, resuspend the collected CTCs in a 1 to 1 ratio of 1x DPBS and reconstituted basement membrane extracted, and keep at 4 °C until the injection. For intravenous injection, resuspend the collected CTCs in DPBS only.

NOTE: Volume of the solution strictly depends on the number of CTCs that will be injected. It is advised to inject in the mammary fat pad or intravenously a maximum of 100 μL per mouse. When calculating the volume, always consider the dead volume for the manipulation and syringe loading.

REPRESENTATIVE RESULTS:

The presented workflow allows the preparation of individual CTCs, either from single CTCs or separated from CTC clusters. CTCs from patients or tumor-bearing mice are enriched from whole blood with available CTC-enrichment methods and then stained with antibodies against cancer-specific markers (e.g., EpCAM, green) and WBC-specific markers (e.g., CD45, red) (**Figure 1A**). The stained CTC product is then transferred to the micromanipulation station were individual cells are picked, deposited in PCR tubes or multiwell plates and prepared for downstream analysis, including single-cell sequencing, in vitro culture or in vivo assays (**Figure 1B**).

Reliability of this method is based on a proper target-cell distinction during manual cell picking. Live immunostaining with anti-EpCAM antibodies visualizes cancer cells in the suspension and enables an accurate distinction from CD45-positive events (most likely, WBCs) (**Figure 2A**). When properly calibrated and maintained, this cell collector provides well-controlled cell manipulation. Precise CTC isolation is characterized by aspiration of only the desired target without surrounding contaminant cells (RBCs or WBCs), as shown on the pictures taken before and after CTC cluster aspiration (**Figure 2B, C**).

As previously described, CTC clusters display a more metastatic phenotype when compared to matched single CTCs[19]. Yet, whether the presence of neighboring cells is sufficient to increase the proliferation rate of cells within clusters is unknown. In order to address this question, 1009 single CTC and 1008 CTC clusters ranging between 2-17 cells (with 89.5% of them being 2-5 cell clusters) derived from the CTC-derived BR16 cell line[20] were micromanipulated into individual wells of 384-well ultra-low attachment plates. The number of live cells in every well was counted

manually under the microscope and recorded weekly. All analyses were performed after normalization of cell number (i.e., the three-cell cluster was analyzed as three individual cells). Unsuccessful colonies were characterized by the lack of living cells in the well at the end of the experiment. As suspected, CTC clusters showed increased survival compared to single CTCs and gave rise to cell colonies within 56 days of in vitro culturing (**Figure 3A, 3B**). Notably, CTC clusters also showed higher proliferation rate and thus reached higher final cell numbers (**Figure 3C**), indicating that direct contact with other tumor cells has an impact on both their viability and proliferation rate.

Lastly, we provide single-cell RNA sequencing data of CTCs directly isolated from breast cancer patients. Particularly, we show a t-Distributed Stochastic Neighbor Embedding (tSNE) of single cells derived from either single CTCs, CTC clusters or CTC-WBC clusters (**Figure 4**). This approach allows the identification of cells with the similar gene expression profile, as well as the distinction of cell populations that differ based on the expression of particular genes.

FIGURE AND TABLE LEGENDS:

Figure 1. Schematic representation of the experimental workflow. (A) CTCs are obtained from the blood of cancer patients or mouse cancer models, enriched using available methods and labeled with antibodies to discriminate tumor cells (*green*) from white blood cells (*red*). **(B)** Precise micromanipulation of CTCs facilitates multiple procedures and applications e.g., single-cell sequencing, CTC culture or in vivo transplantation experiments.

Figure 2. Representative pictures of CTCs before and after micromanipulation. (A) Representative images of CTCs derived from a CTC-derived xenograft (NSG-CDX-BR16) and stained with antibodies anti-EpCAM (*green*) and anti-CD45 (*red*) to enable the visualization of CTCs and white blood cells, respectively. Magnification 40x is shown. **(B,C)** Micromanipulation allows for the precise separation of CTCs from unwanted cells as shown in the images before (*left*) and after (*right*) cell-aspiration procedure. Magnification 10x. The larger field of view **(B)** and narrower field of view **(C)** are shown, respectively.

Figure 3. Survival and proliferation analysis of single CTCs and CTC clusters. Individual cells from single CTCs or CTC clusters from cultured CTC-derived BR16 cells were micromanipulated into 384-well plates. **(A)** Kaplan-Meier plot showing the survival probability of seeded single cells versus cell clusters. $P<0.0001$ by pairwise Log-Rank test. **(B)** Bar graph representing the proportion of colonies that consisted of live cells at the end of the experiment (day 56). $P<0.0001$ by pairwise Log-Rank test. **(C)** Heatmaps visualizing normalized cell number distribution over the course of the experiment (day 0, 8, 32, 56). Each block represents one starting cell and the heatmap shows the number of cells per well at a given timepoint. d=day.

Figure 4. Single-cell RNA sequencing. Visualization of CTC RNA expression data using t-Distributed Stochastic Neighbor Embedding (tSNE). Each dot represents a single cell derived from a single CTC, a CTC cluster or a CTC-WBC cluster. Colors of the dots correspond to the donor ID.

DISCUSSION:

The molecular characterization of CTCs holds the promise to improve our understanding of the metastatic process and guide the development of new anti-metastasis therapies. Here we provide a detailed description of those protocols that enable CTC micromanipulation and downstream analysis, including both single cell-based functional assays, gene expression analysis and in vivo transplantation for metastatic potential assessment[20].

Among the most critical steps of our protocol, micromanipulation of CTC-enriched products aims at gaining single cell resolution from relatively heterogeneous cell suspensions, i.e. allowing to reach the highest levels of purity and to improve the quality of subsequent functional or molecular analyses. For example, single cell picking of CTCs has enabled us and others to investigate CTC heterogeneity (e.g., differences between single CTCs, CTC clusters, and CTC-WBC clusters), both from a molecular standpoint and from the perspective of being able to assess metastasis-initiation capability. While we generally favor cell picking protocols that allow the experimenter to manually isolate CTCs (i.e. allowing for a higher degree of flexibility depending on the characteristics of individual targets), automated solutions are now available to facilitate cell picking and to accelerate the CTC isolation process in well-controlled experiments.

When considering single cell micromanipulation in the context of CTC analysis, time is a very critical limiting factor. Since our protocol is meant to be conducted on living cells, it is imperative to proceed as fast as possible to minimize changes due to the ex vivo environment, such as the upregulation of downregulation of genes that are context-dependent. When compared to the existing techniques for CTC analysis, single cell micromanipulation of live CTCs offers higher flexibility for the downstream analysis of choice, ranging from single cell sequencing to direct functional assays.

In this manuscript, we also provide new data that highlight important differences between single and clustered CTCs by micromanipulation and seeding more than thousand single CTCs or CTC clusters (with a clearly defined size) from a CTC-derived cell line in individual wells of a microtiter plate. First, we observe that CTC clusters (i.e., the presence of neighboring cells) are sufficient to achieve better survival rates of seeded cells, supporting our in vivo data that highlight lower apoptotic rates of CTC clusters upon seeding at a distant site[19]. Further, even upon normalization for the number of seeded cells, cancer cells grown as clusters display much higher proliferation rates, further reinforcing the concept that clustered CTCs are highly efficient metastasis contributors.

Together, we present specific protocols for CTC analysis with the purpose to promote single cell-related investigations in the CTC field. In the future, we anticipate that these protocols might be useful for CTC-related investigations, aiming towards a better understanding of the biology that characterizes blood-borne metastasis in various cancer types.

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DISCLOSURES:

N.A. and B.M.S. are listed as inventors in patent applications that relate to circulating tumor cells and the treatment of cancer. N.A. is a paid consultant for pharmaceutical and insurance companies with an interest in liquid biopsy.

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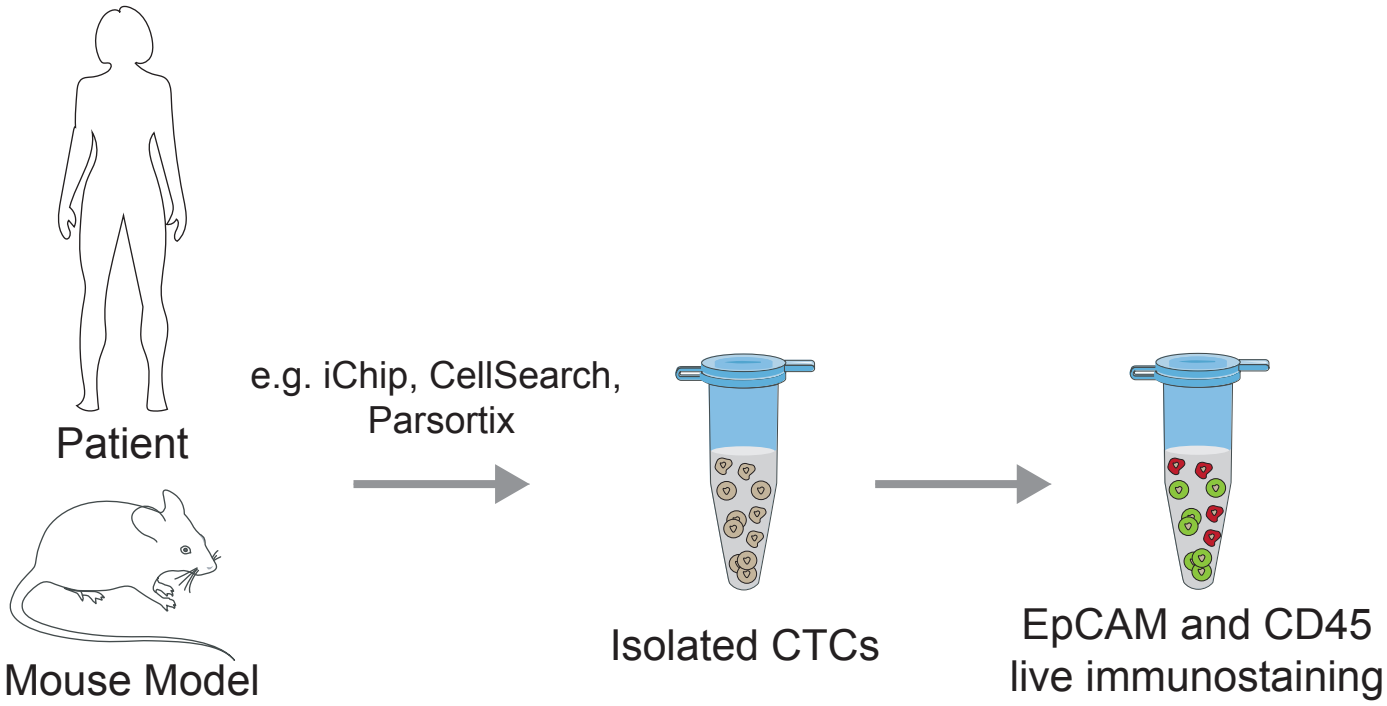
A



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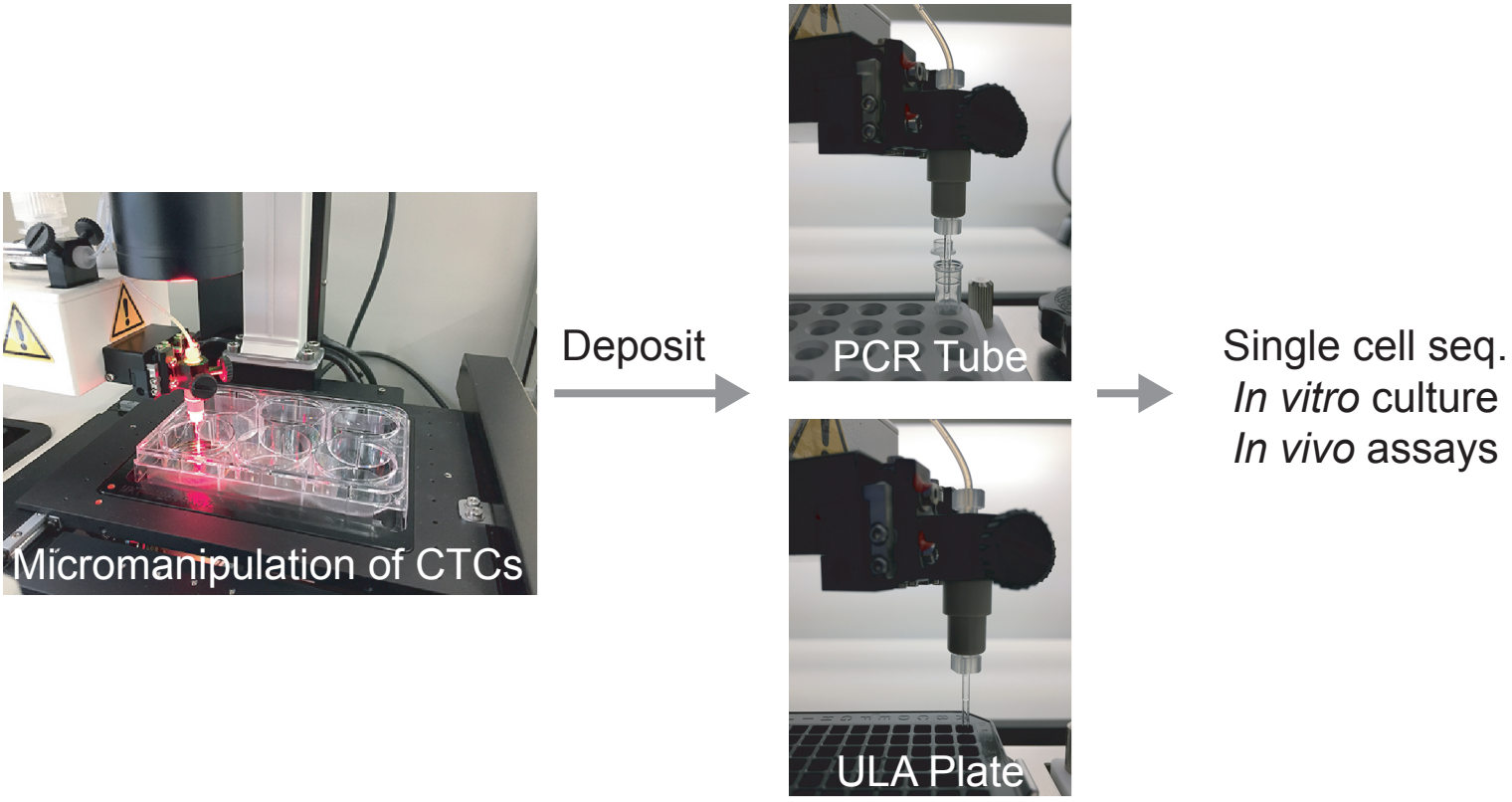

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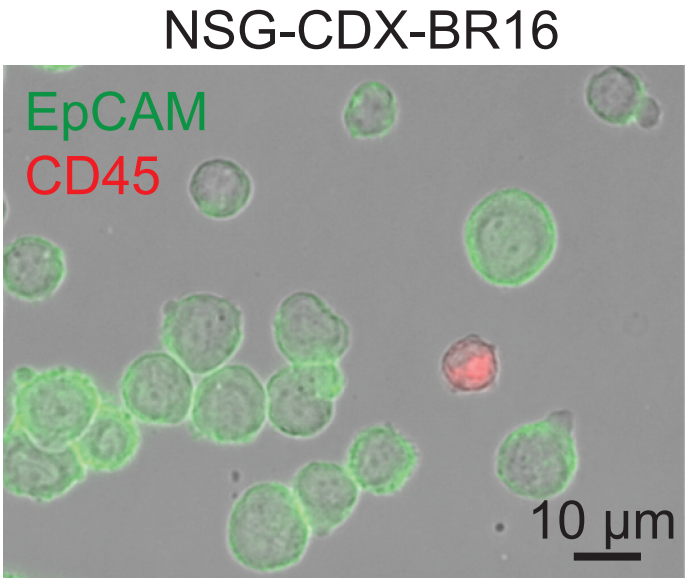

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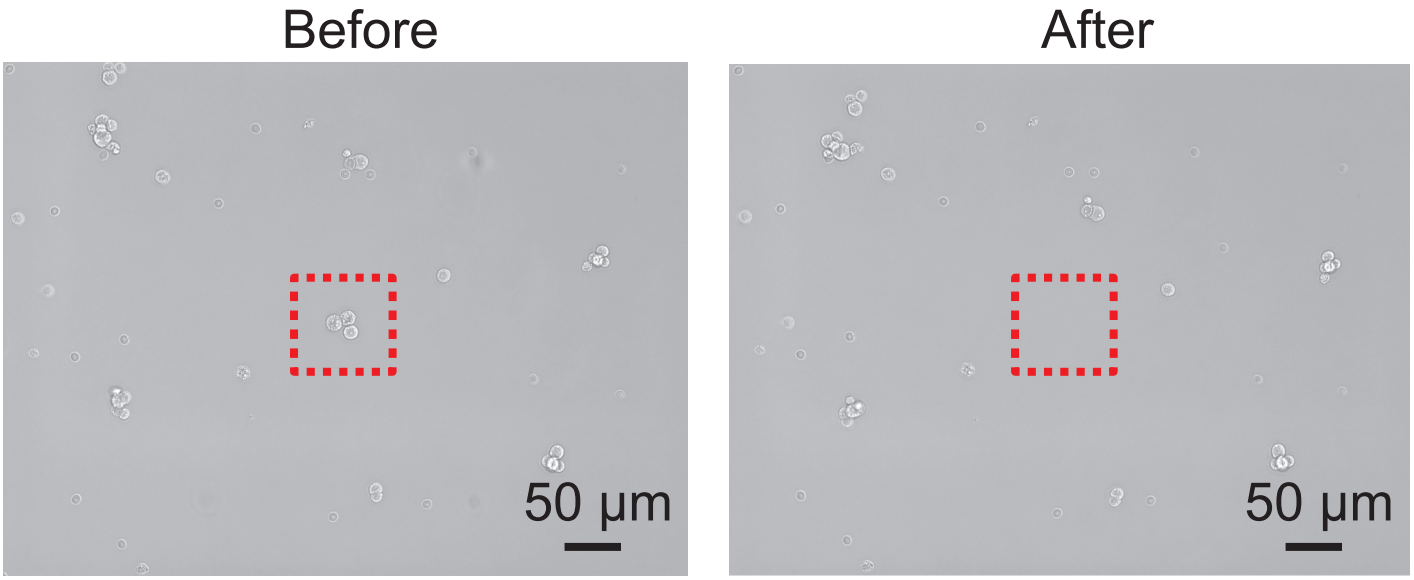

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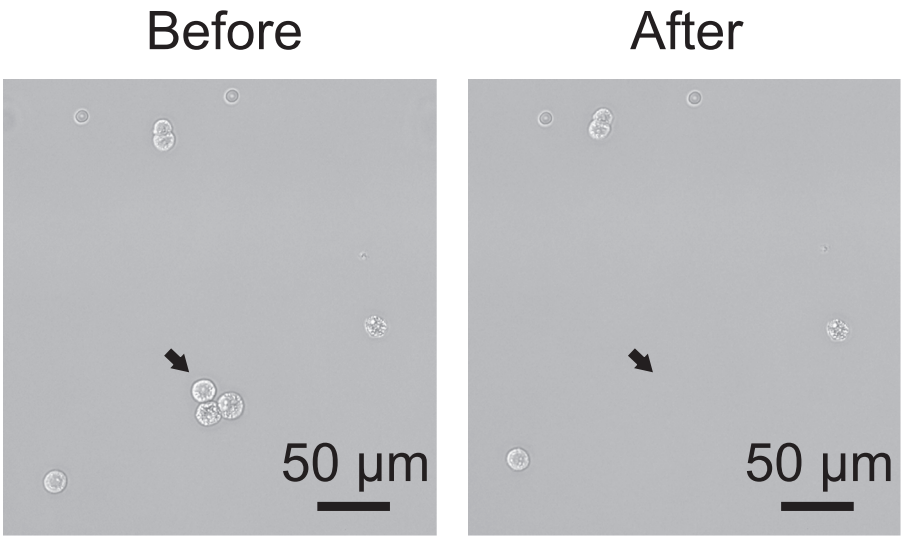

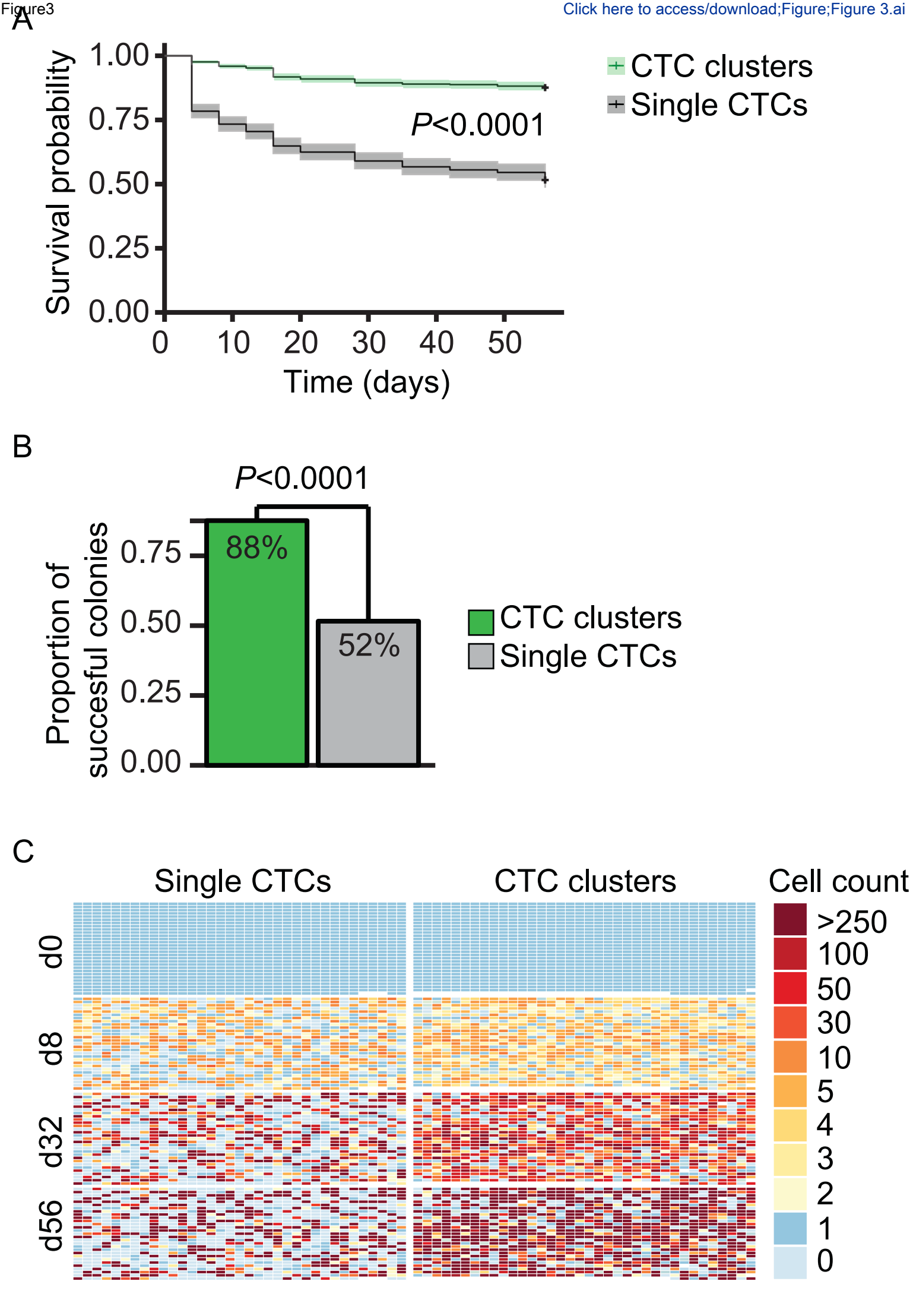

Figure4

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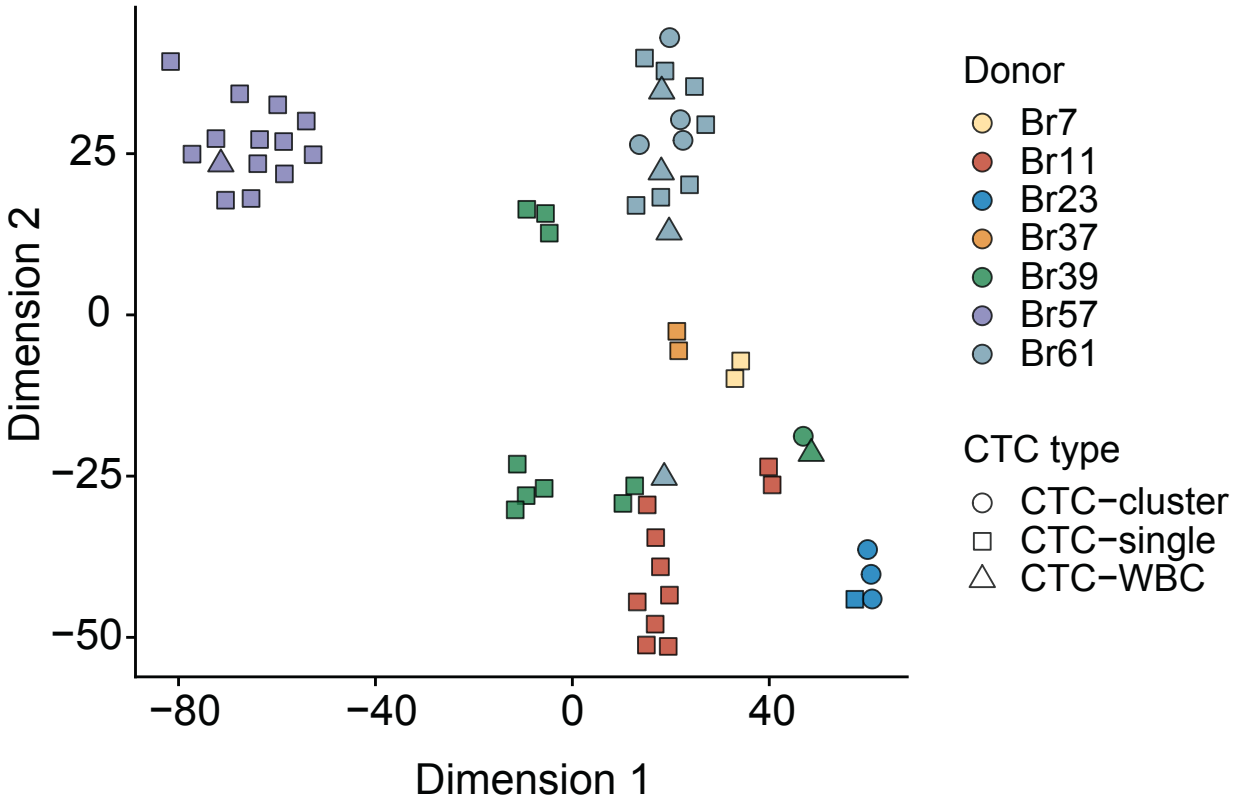

Name of Material/ Equipment	Company
Anti-human EpCAM-AF488	Cell Signaling Technolo
1X DPBS	Invitrogen
6-wells Ultra-low attachment plate	Corning
Anti-human CD45-BV605	Biolegend
Anti-human EGFR-FITC	GeneTex
Anti-human HER2-AF488	Biolegend
Anti-mouse CD45-BV605	Biolegend
BD Vacutainer K2EDTA	BD
Cell Celector	ALS
CellD software	ALS
Cultrex PathClear Reduced Growth Factor BME, Type	R&D Systems
Micro tube 1.3 mL K3EDTA	Sarstedt
PCR tubes	Corning
RLT Plus	Quiagen
SUPERase In RNase Inhibitor	Thermo Fisher

Catalog Number	Comments/Description
CST5198	clone: VU1D9
14190169	no calcium, no magnisium
3471	
304041	clone: HI30
GTX11400	clone: ICR10
324410	clone: 24D2
103139	clone: 30-F11
366643	for human blood collection
CC1001	core unit
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41.3395.005	for mouse blood collection
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AM2696	1 U/µL

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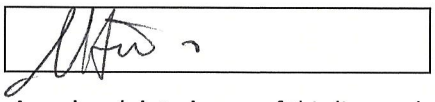
Signature:	
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