Chapter 9

Vita-Assay™ Method of Enrichment and Identification of Circulating Cancer Cells/Circulating Tumor Cells (CTCs)

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Abstract

The ability to capture, enrich, and propagate circulating cancer cells/circulating tumor cells (CTCs) for downstream analyses such as ex vivo drug-sensitivity testing of short-term cultures of CTCs, single cell sorting of CTCs by fluorescence activated cell sorting (FACS), animal injection tumor and/or metastasis formation studies, next generation sequencing (NGS), gene expression profiling, gene copy number determination, and epigenomic analyses is of high priority and of immense importance to both the basic research and translational/clinical research communities. Vitatex Inc.'s functional cell separation technology, constructed as Vita-Assay™ (AG6W, AN6W, AR6W) culture plates, is based on the preferential adhesion of invasive rare blood cells of tissue origin to a tissue or tumor microenvironment mimic—the so-called cell adhesion matrix (CAM), which has a demonstrated ability to enrich viable CTCs from blood up to one-million fold.

The CAM-scaffold allows for the functional capture and identification of invasive CTCs (iCTCs) including invasive tumor progenitor (TP) cells from cancer-patients' blood. CAM-captured CTCs are capable of ingesting the CAM (CAM+) itself. Green and red fluorescent versions of Vita-Assay™ (AG6W and AR6W) allow for direct visualization of CAM-uptake by cancer cells. Vita-Assay™ CAM-enrichment has allowed for sensitive multiplex flow cytometric and microscopic detection of iCTCs from patients with cancers of the breast, ovary, prostate, pancreas, colorectum, and lung; it has also been successfully utilized for ex vivo drug-sensitivity testing of ovarian-cancer patient CTCs. The CAM enrichment method is equally suitable for the separation of iCTCs and TP cells in ascites and pleural fluid.

Key words Cancer cell capture, Cell invasion, Metastasis detection, Rare-cell enrichment, Circulating cancer cells, Circulating tumor cells, iCTCs, Vita-Assay™, Cell-adhesion matrix, Vitatex Inc

1 Introduction

The development of human cancer is a multistep process driven by genome instability which ultimately results in the acquisition of at least eight major biological capabilities of cancer cells, namely, sustained proliferative signaling, evasion of growth suppressors, an increased resistance to cell death, an unlimited replicative potential (immortality), the ability to induce angiogenesis, evasion of

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immune destruction, a reprogramming of energy metabolism, and activation of the multistep process of invasion and metastasis (invasion–metastasis cascade) [1, 2].

Metastasis, or dissemination of primary tumor cells ("seeds") through the blood and lymph systems to distant secondary-sites/organs ("soil") in the body, involves a cascade of sequential and discernible steps including the loss of cellular adhesion; an increase in cell motility; invasion of adjacent tissues; entry into, survival in, and transport through the circulation; arresting in a secondary site; and eventual extravasation and growth in the new tissue/organ [3–7]. Indeed, it is this very process, cancer metastasis, which is responsible for more than 90 % of cancer-associated deaths [5, 8–11]. Despite encompassing such a lethal series of steps in neoplastic disease progression, experimental models indicate that of the millions of cancer cells that escape the primary tumor and are continuously being dispersed through the body, only a small number of these cells (<0.1 %) are able to survive in the circulation (viable CTCs), reach a distant organ, survive in a dormant state in the secondary site, evade the immune system and any systemic therapy a cancer patient is receiving, and grow into an overt metastatic lesion [7, 12–17].

It should therefore come as no surprise the immense interest shown by cancer biologists and clinical oncologists alike and the impetus being put on capturing, enriching, identifying, and studying these extremely rare, viable, metastasis-initiating CTCs/cancer stem/tumor progenitor cells, the key players in the transition from localized to systemic disease [4, 7, 9, 13, 18]. Basic cancer research studies in areas such as the molecular characterization of CTCs as well as clinical applications such as patient CTC enumeration and sequential CTC-monitoring of cancer patient blood during systemic treatment are of high value and importance. The main obstacle in studying CTCs is of course the rarity that they appear in blood at approximately one CTC in a background of a billion red blood cells, and millions (10^6–10^7) of white blood cells and at an average frequency on the order of 10–100 CTC per mL of whole blood [13, 19–22].

The Vita-Assay™ CTC-enrichment platform has been specifically designed to functionally capture and enrich (up to one million-fold) these rare, viable, and invasive CTCs (iCTCs) and to exploit their preferential adherence to the CAM surface attributable to their inherent invasive nature and high avidity for the extracellular matrix (ECM), with the added advantage that CAM-captured CTCs can be identified after subsequent ingestion of fluorescent-CAM (CAM^+) [18, 23–26]. Furthermore, since the functional proclivities to degrade and ingest the ECM are major acquired capabilities of invasive and metastatic cells, CAM^+ cells represent a unique way to identify and enrich CTCs, and in all likelihood these iCTCs encompass resident TP or cancer stem cells [18, 23–26].

The CAM method allows for the capture of iCTCs indiscriminant of tumor cell type (primary tumor origin), cell size, CTC morphology or expression of particular protein-markers and thus offers a robust, comprehensive opportunity to capture true metastasis-initiating cells. Vita-Assay™ has allowed for sensitive multiplex flow cytometric and microscopic detection of CTCs/iCTCs from patients with cancers of the breast, ovary, prostate, pancreas, colorectum, and lung to date, and has been used to relate CTC enumeration and prognosis, as well as to generate genotypic and phenotypic data from cultured CTCs captured from the blood of prostate, ovarian, and breast cancer patients [18, 23–26]. Since patient's CTCs captured using Vita-Assay™ plates are viable, continued culture of CTCs in the same plate is suitable for ex vivo drug-sensitivity and resistance testing.

In conclusion, the Vita-Assay™ method enables the efficient capture, enrichment, and identification of rare, invasive, and viable CTCs, allowing for an array of downstream analyses/applications of both cancer research (CTC culturing, mutational analysis, molecular characterization, ex vivo drug selection studies, etc.) and clinical investigation (CTC enumeration, prognostic value, and sequential measurement of patient CTC number to monitor treatment response).

2 Materials

2.1 Vita-Assay™

1. Vita-Assay™ plate (Vitatex, Stony Brook, NY, USA): Each 6-well plate is coated with either plain-CAM (Vita-Assay™ AN6W, Product # 102.01 N) or red fluorescent-CAM (Vita-Assay™ AR6W, Product # 102.02R) or green fluorescent-CAM (Product # 102.03G). Each plate is equivalent to six assays and allows for the processing of six distinct patient samples on a single plate (*see* **Note 1**).
2. Each Vita-Assay™ also includes three 100-μL tubes of Cell Releasing CAM Enzyme (*see* **Note 2**). Cell Releasing CAM Enzyme must be stored in a freezer (−20 °C) immediately upon receipt (*see* **Notes 3** and **4**).

2.2 Necessary Materials Not Supplied with Vita- Assay™

1. BD Vacutainer® 10.0 mL sodium heparin tubes are recommended (*see* **Note 5**).
2. Antibodies for Flow Cytometry: Positive identification of iCTCs: Use (1) PE-conjugated TP marker (PE-anti-seprase/PE-anti-CD44 antibody mix (Vitatex). Alternatives are (2) PE-EpCAM (BioLegend), or antibodies against (3) CA125 (ovarian), (4) CA19-9 (pancreatic), (5) HER2 (breast), (6) PSMA (prostate). For exclusion of hematopoietic lineage (HL) or immune cells, use APC-conjugated anti-CD45 (BD).

3. Antibodies for Microscopy: Positive identification of iCTCs: FITC-conjugated TP marker (FITC-anti-seprase/anti-CD44 antibody mix (Vitatex) or FITC-anti-Epi mix of ESA (Biomeda), EPCAM (BioLegend), and BerEp4 (Dako). For exclusion of hematopoietic lineage (HL) or immune cells, use mouse anti-human CD45 (BD).
4. Dako color reaction Kit: (1) secondary antibody-biotinylated link, (2) streptavidin-AP, (3) BCIP/NBT substrate system.
5. 7-aminoactinomycin D (7-AAD).
6. Hoechst 33342 or DAPI.
7. 10× BD FACS Lysing solution (BD).
8. A low-speed, swing-out bucket centrifuge for pelleting cells.
9. A temperature-controlled CO_2 incubator.
10. A vacuum aspirator.
11. Sterile 15-mL polystyrene conical centrifuge tubes.
12. Sterile 50-mL polystyrene conical centrifuge tubes.
13. 5-mL polystyrene tube with cell strainer cap.
14. 10× Red cell lysis buffer: 1.54 M NH_4Cl, 100 mM $KHCO_3$, 1 mM EDTA pH 8.0. Either prepare or purchase commercially available RBC Lysis Buffer 10× (BioLegend).
15. Standard complete medium containing 10 % fetal calf serum. Alternatively, complete cell culture (CCC) medium: 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and RPMI 1640 supplemented with 10 % calf serum, 5 % Nu-serum, 2 mM L-glutamine, 1× penicillin–streptomycin Solution.
16. Sterile 1× phosphate buffered saline (PBS), pH 7.4.
17. Sterile 1× phosphate buffered saline containing **Ca2+ (Ca2+ PBS)**, pH 7.4.
18. Sterile 0.2 % bovine serum albumin (BSA) in 1× PBS, pH 7.4.
19. Adjustable water bath set to 37 °C.
20. Cytocentrifuge system (such as Statspin cytofuge).
21. Trypsin–EDTA solution.
22. Standard fluorescent microscope system with phase-contrast, blue, green, and red channels.

3 Methods

Carry out procedures at room temperature and under sterile conditions unless otherwise specified.

3.1 Vita-Assay™: Cell Isolation Protocol

1. Blood Collection: Using your institution's recommended procedure for blood standard venipuncture, collect blood into one or more BD Vacutainer® 6.0 mL sodium heparin tubes or 6.0 mL lithium heparin tubes or BD Vacutainer® 10.0 mL sodium heparin tubes. Transport tubes to laboratory (*see* **Notes 6** and **7**).
2. Preparation of the nuclear cell fraction by red blood cell (RBC) lysis: For preparation of nuclear cells from whole blood, red blood cells in the blood samples will be lysed by mixing blood and 1× RBC Lysis Buffer at a ratio of 1:25 at 20–25 °C. For a 2-mL blood sample, mix 2-mL blood and 48-mL 1× RBC Lysis Buffer at 20–25 °C in a sterile 50-mL conical tube.
3. Rotate the 50-mL tube with blood mix on a rotator at low speed (10 rpm, Stovall Low Profile Roller) for 5 min at 20–25 °C.
4. Pellet nuclear cells by centrifugation to 350×*g* for 5 min. Carefully remove supernatant.
5. Add 4-mL of a Complete Cell Culture (CCC) medium to each 50-mL conical tube to resuspend the cell pellet, resulting in a total of 4-mL cell suspension in CCC medium containing the cells derived from 2-mL whole blood.
6. Loading cell suspension into wells of Vita-Assay™ plates: Aliquot 2-mL of the cell suspension into each of the 6 wells of the Vita-Assay™ plate coated with CAM. Culture cells in a 5–7 % CO_2 incubator at 37 °C for 1 h to allow adherence of tumor cells into CAM to obtain the enriched CAM-avid cells.
7. Wash away floating cells and culture CAM-avid cells: After 1 h incubation above, remove unattached cells and existing medium and discard into a waste container.
8. Wash wells one time by pipetting 2-mL of CCC medium gently down the side of each well.
9. Move the plate in a horizontal circle six (6) times and discard wash solution.
10. Add 2-mL of CCC medium into each well and culture cells for 18 h to enhance signal of CAM uptake by iCTCs (only AG6W and AR6W will display CAM uptake signal) (*see* **Notes 8** and **9**).
11. After 18 h cell incubation in each well remove any unattached cells along with medium into a waste container.
12. Freshly prepare CAM enzyme working solution by diluting the provided frozen enzyme with 2-mL of 1× phosphate buffered saline containing Ca^{2+} (Ca^{2+} PBS), pH 7.4 for use in **step 14** (*see* **Note 10**).

13. Wash wells three times by pipetting 2-mL of 1× PBS into each well each time, followed by moving the plate in horizontal circle three (3) times and discarding the wash solution.
14. Add 1-mL of the CAM enzyme working solution into each washed Vita-Assay™ well.
15. Place Vita-Assay™ plate in a CO_2 incubator at 37 °C for 10 min to enzymatically dissolve CAM and release tumor cells into suspension.
16. Optional Step: Skip this step when processing cancer-patient blood samples. Only follow this step for experiments in which cancer cell-lines are spiked-in to healthy donor blood (CTC model experiments) and only if CAM-enzyme alone does not detach tumor cells, which are usually two to four times bigger than co-isolating hematopoietic cells. Add 1-mL of the trypsin–EDTA solution into each Vita-Assay™ well, and place in a CO_2 incubator at 37 °C for 5 min to detach remaining adherent cells.
17. Transfer cell suspension into a new 15 mL conical centrifuge tube.
18. Wash the well (sequentially with other wells of the same experimental condition) of Vita-Assay™ plate one (1) time with 3-mL of CCC medium.
19. Transfer the wash into the 15 mL tube with enzymatically released cells (*see* **Note 11**).
20. Concentrate cells by centrifuging the 15 mL conical tube at 350 × *g* for 5 min.
21. After centrifugation, remove supernatant by gentle aspiration, retaining the last 100 μL containing the enriched cell fraction (*see* **Note 12**). The 100 μL cell suspension can be processed for cellular analyses including, but not limited to, enumeration by automated flow cytometry (Subheading 3.2 that follows below); validation of cell identity by microscopy (Subheading 3.3 below); and CTC cell culture (Subheading 3.4 below).

3.2 CTC Enumeration Using Automated Flow Cytometry

1. From **step 21** of Subheading 3.1 above, loosen and resuspend the cell pellet by pipetting up and down five times.
2. Fixation: Fix cells by adding 1.0 mL of 1× BD FACS Lysing solution to the 100 μL of cell suspension and incubating at 20–25 °C for 10 min.
3. After incubation in fixative, add 3-mL of PBS containing 0.2 % BSA, mix and concentrate cells by centrifuging the 15 mL conical tube at 350 × *g* for 5 min.
4. After centrifugation remove supernatant by gentle aspiration, retaining the last 80 μL containing the enriched fixed-cells. The fixed cell suspension could be stored at 2–8 °C at this point.

5. Antibody and nucleic acid dye staining: Add 6 μL PE-anti-seprase/CD44 antibody cocktail (or 6 μL PE-anti-Epi), 10 μL APC-anti-HL antibody and 10 μL of 50 μg/mL stock of 7AAD to the 80 μL cell suspension and stain cells at 20–25 °C in the dark, for 30 min.
6. Washing of stained cells: Add 3-mL of PBS containing 0.2 % BSA.
7. Collect cells by centrifugation at 350 × *g* for 5 min.
8. Remove supernatant and save the last 500-μL containing fixed, stained cell suspension in sterile PBS containing 0.2 % BSA.
9. Preparation for flow cytometric cell counting: Particulates in fixed, stained cells must be filtered away using a polystyrene tube with cell strainer cap. Collect cells by centrifugation at 350 × *g* for 5 min.
10. Count cells with a multiplex flow cytometer, i.e., BD FACSCalibur™ (*see* **Note 13**).
11. Reporting: Flow cytometry plots and/or tables of enumeration of CTCs and/or iCTCs and immune cells per 1-mL of blood are shown (Fig. 1).

3.3 Validation of CTC Identity (on Vita-Assay™ AR6W) by Microscopy

The number of CTCs or iCTCs ascertained by flow cytometry can be validated using microscopy for cellular morphology of CTCs as compared to immune cells, observed as positive for CAM uptake (CAM+) and Epi or TP expression, and negative for hematopoietic lineage (HL) markers, as described in Protocol below. The microscopy step offers a clear morphological discrimination of tumor cells from hematopoietic (immune) cells.

1. Microscopy—Validation of iCTCs, CAM-avid CTCs, and immune cells: From **step 21** of Subheading 3.1, Loosen the cell pellet by pipetting up and down five times.
2. Fixation: Fixation can be done by adding 100 μL of 2 % paraformaldehyde in PBS, pH 7.3, to the 100 μL cell suspension, and incubate at 20–25 °C for 5 min.
3. Add 3-mL of PBS containing 0.2 % BSA, mix and concentrate cells by centrifuging the 15 mL conical tube at 350 × *g* for 5 min, and remove supernatant by gentle aspiration, retaining the last 100 μL containing the enriched cells. The fixed cell suspension could be stored at 2–8 °C at this point.
4. Antibody and nucleic acid dye staining: Add 8 μL anti-HL antibody mix to the 100 μL cell suspension and incubate for 20 min, followed by staining using Dako kit: secondary-antibody biotin-conjugate, Streptavidin-AP enzyme, and BCIP/NBT substrate according to manufacturer's specifications (positive cells will stain blue/purple). Wash 1× in PBS containing 0.2 % BSA, and add 8 μL of FITC-TP mix or FITC-Epi mix, and add 1 μL 300 μM DAPI (or equivalent Hoechst) and stain cells at 20–25 °C in the dark, for 30 min.

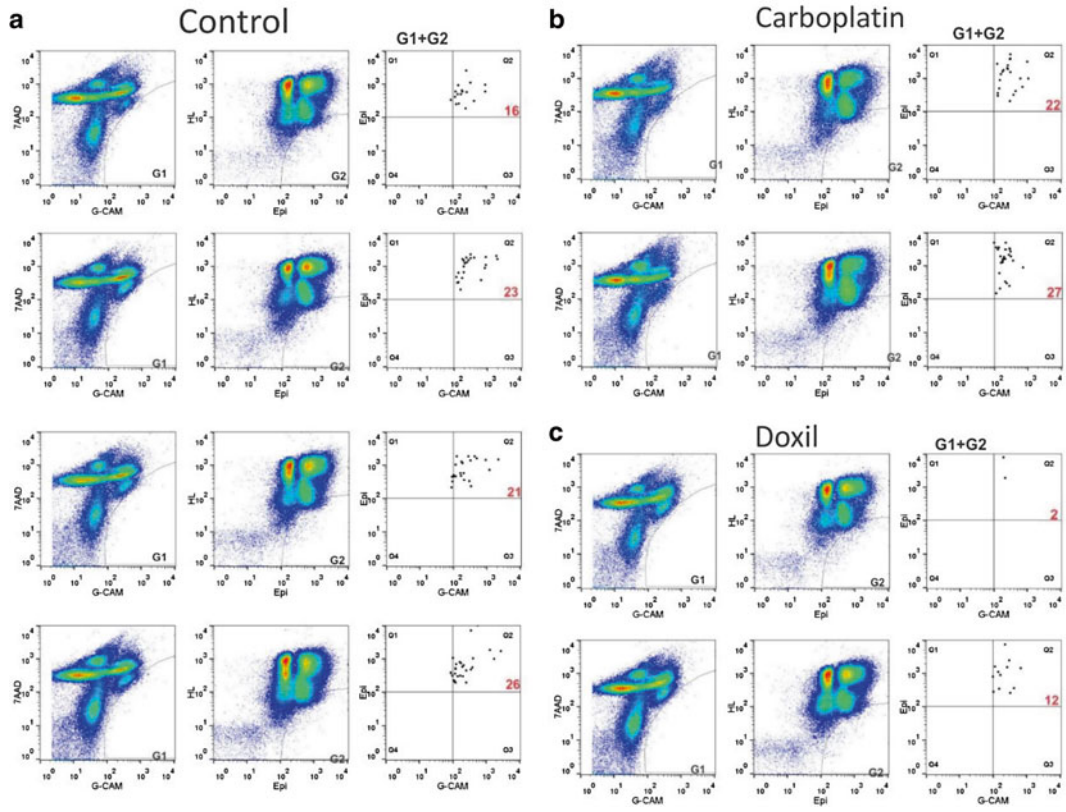

Fig. 1 Flow cytometric analysis and enumeration of iCTC and immune cells captured on Vita-Assay™ AG6W from 0.67 mL blood of a recurrent ovarian cancer patient (SB448-1). Cells were cultured (ex vivo) for up to 72 h in the absence (control) (**a**), or presence of chemotherapeutic drugs demonstrating drug-resistance (approximately equal counts of iCTCs) with (**b**) Carboplatin or indicating drug-sensitivity (lowered iCTC counts) with (**c**) Doxil (Doxorubicin). Gates were made on G-CAM^{+} (CAM uptake)/7AAD^{+} fixed cells (G1) to exclude platelets or non-cellular particles (*left panel*, events on 10^{0} 7AAD) that also exclude HL$^{+/dim}$ events (G2). Events that overlap between G1 and G2 are identified and enumerated as iCTC counts shown in *red* on the *right panel* of the plot for each blood sample. Immune cells were shown as 7AAD+ HL+ clusters in *left* two panels. Note that none of the experiments exhibited artifacts that were not possible to analyze; accordingly, the assay failure rate for the iCTC flow cytometry detection is estimated as 0 %. When cross samples were compared, iCTC and immune cell counts were standardized to per 1-mL blood counts

5. Washing of stained cells: Add 3-mL of sterile PBS containing 0.2 % BSA. Collect cells by centrifugation at 350 × *g* for 5 min. Remove supernatant and save the last 200-μL containing fixed, stained cell suspension in sterile PBS containing 0.2 % BSA.
6. Preparation for microscopy: To concentrate isolated cells to 7-mm diameter area on a microscopic glass slide for microscopic analysis, cytospin preparation should be performed using devices, i.e., StatSpin cytofuge and Filter Concentrators.
7. After concentrating cells on glass slide, place a drop of mounting medium on the cell sample on the microscopic slide.

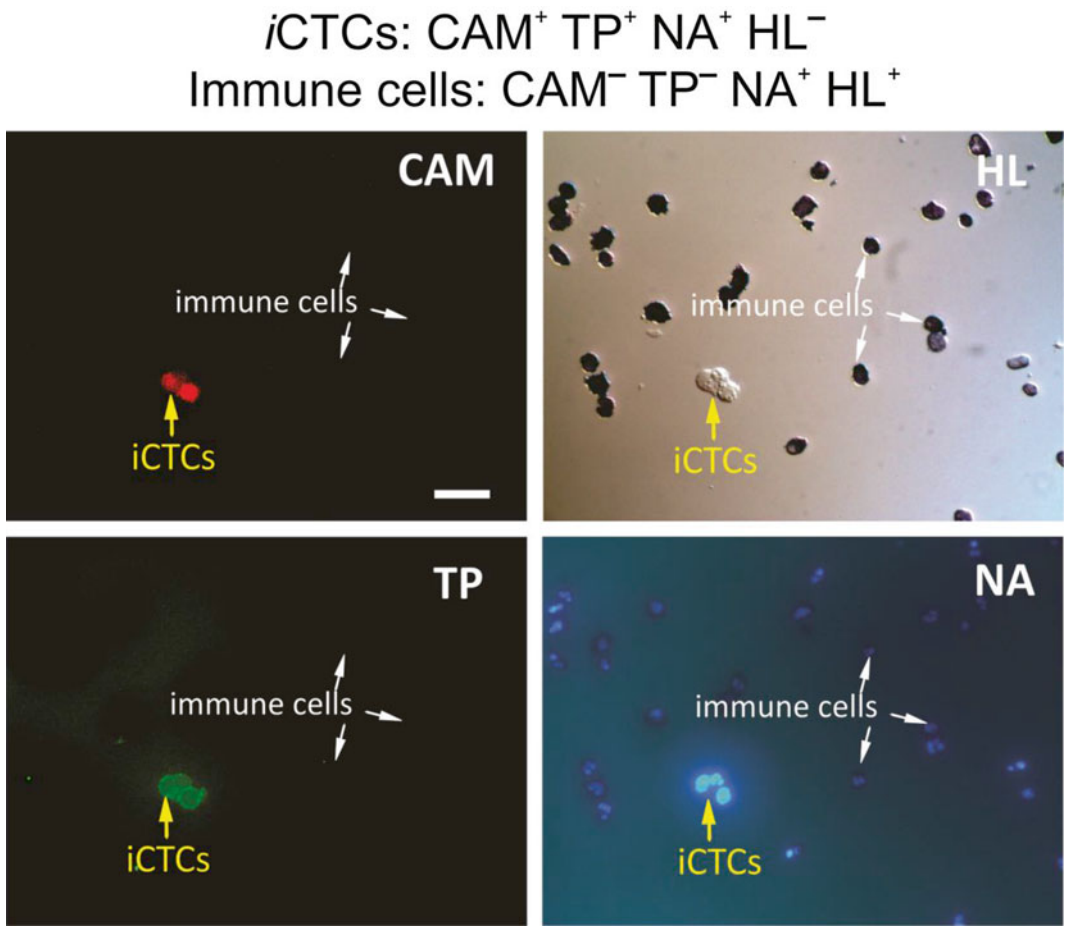

Fig. 2 Validation of CAM+ flow cytometric isolation of iCTCs by microscopic imaging. Phase contrast microscopy and fluorescence microscopy were used to image cells from 1 mL of blood of an ovarian cancer patient (SB423-2) which were captured and identified using Vita-Assay™ AR6W. In this field three iCTCs were identified and observed to be CAM+ (invaded into and ingested *red-fluorescent* CAM), TP+ (CD44+/seprase+) and NA+ (nucleic acid using Hoechst 33342). Take note of the numerous hematopoietic lineage cells (HL+) stained with *blue* color substrate-conjugated secondary antibody against primary CD45 antibody, and were unable to ingest CAM (CAM negative), and were also tumor/tumor progenitor marker negative (TP negative). Bar in CAM panel =40 μm

Examine and record images of cell types (CAM+, TP+, NA+, CD45−) using a fluorescence microscope equipped with multiple filters (Fig. 2).

3.4 CTC Cell Culture for Downstream Applications

CTCs in blood and captured in the Vita-Assay™ plate can be propagated in culture in the same plate in CCC media for a period of at least 14 days without loss of cellular viability, whereas the majority of co-isolating hematopoietic cells are lost within 7 days (Fig. 3). The culture of viable CAM-avid tumor and immune cells for a period of time allows for sufficient expansion of CTCs for use in a

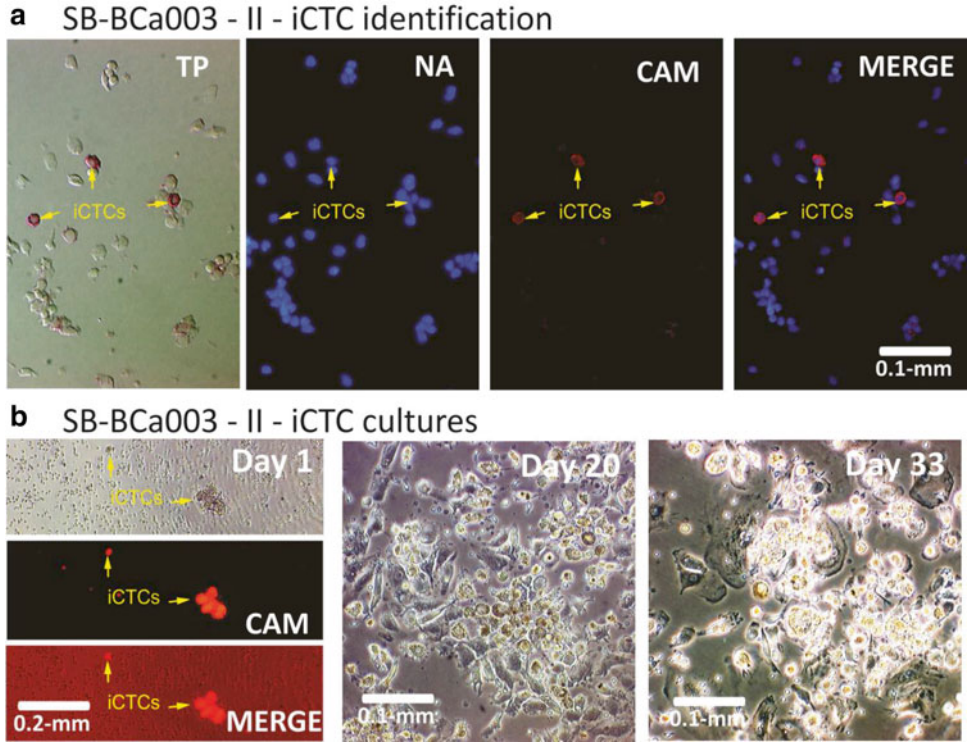

Fig. 3 (**a**) Fluorescence microscopic images of cells captured on Vita-Assay™ AR6W and derived from 1 mL blood of a patient with stage II breast cancer (SB-BCa003-II). iCTCs were identified as TP (anti-CD44/anti-seprase) positive and were stained with *red* color substrate-conjugated secondary antibodies against primary TP antibodies that targeted iCTCs, in addition to being nucleic acid positive (NA) as determined with Hoechst 33342 dye+, and CAM+ having ingested red-fluorescent CAM. Single *yellow arrows* depict iCTCs. (**b**) Vita-Assay™ AR6W CAM-enriched cells were cultured in CCC media on the CAM scaffold for 1–33 days. Live iCTCs were photographed under phase contrast microscopy and fluorescence microscopy (red-fluorescent CAM, to reveal CAM uptake/labeling of tumor cells). iCTCs propagated as time increased. On day 1 (**b**, *left panels*), tumor cells (iCTCs) were seen to associate with and ingest/uptake CAM and morphologically presented as solitary cells and in a cluster (*yellow arrows*) larger than hematologic cells (smaller cells seen under phase). By day 20 (**b**, *middle panel*), iCTCs were observed to have propagated and expanded in culture, whereas co-isolating hematologic cells decreased in number and were not apparent in the field. By day 33 (**b**, *right panel*), tumor cells grew in clusters with large epithelioid cells

variety of downstream applications, for example, to harvest cells and isolate DNA, RNA, or protein for CTC mutational and/or molecular analyses. In addition, viable CAM-avid tumor cells can be applied in ex vivo drug sensitivity testing and mouse injection/tumor formation studies that are intended for improved therapeutic intervention. Simply follow **steps 1–10** of Subheading 3.1. Cells from that point can be cultured on CAM in CCC media. Below is a brief overview of the procedure if testing out an inhibitor or drug on CTCs in culture.

1. Blood Collection/Shipping: For culture and experimental testing of viable CTCs a larger volume (for example 10–20 mL

or if possible more) of blood is preferred. Venous blood will be drawn from a cancer patient using three BD Vacutainer® 10.0 mL green capped, sodium heparin tubes. Blood samples can be collected in the clinic and shipped using overnight express shipping from distant sites at refrigerated temperature (2–8 °C) to lab.

2. Red Blood Cell Lysis: Prepare $1–10 \times 10^6$ nuclear cells from 1-mL blood using red blood cell lysis buffer.
3. Suspend cells in 2-mL CCC medium and seed cells for 1 h in 1 well of Vita-Assay™ plate.
4. CTC Enrichment and Experimental Testing: After the 1 h incubation, remove floating cells and replace fresh medium ± inhibitor/drug/or any combination thereof in each well of Vita-Assay™ plates. It is highly recommended to conduct such studies in duplicate if not triplicate (*see* **Note 14**).
5. Flow Cytometry—Cell Identification and Enumeration: After at least 2 days in culture, rotate and wash the cellular layer 3× with PBS.
6. Perform enzymatic digestion of CAM using CAM-enzyme.
7. Collect CAM-bound cells, fix cells, and stain cells with APC-anti-hematopoietic lineage (HL) antibody markers, PE-anti-TP antibody markers, and nucleic acid (NA) dye 7AAD for enumeration of iCTCs and immune cells by flow cytometry (*see* **Note 15**).

4 Notes

1. One Vita-Assay™ plate can be used for six assays or six rare cell separations. $1–15 \times 10^6$ nuclear cells from 1–3 mL of whole blood (after red blood cell lysis) should be loaded into 1 well of the 6-well plate.
2. CAM-enzyme is used for enzymatic digestion of CAM and release of CTCs/endothelial progenitor cells from CAM.
3. Immediately prior to use, dilute the frozen cell releasing CAM enzyme by adding 2-mL of 1× phosphate buffered saline containing Ca^{2+} (Ca^{2+} PBS), pH 7.4, to make CAM enzyme working solution.
4. Any unused CAM-enzyme working solution can be stored at 2–8 °C for up to 5 days.
5. Other suitable recommended tubes are BD Vacutainer® 6.0 mL sodium heparin tubes (BD catalog # 367878) or 6.0 mL lithium heparin tubes (BD catalog # 367886).
6. Blood in collection tubes may be stored for up to 48 h at 2–8 °C prior to cell enrichment steps.

7. All pipetting steps from this point should be conducted in a laminar flow hood using sterile technique.
8. The 6 wells can be used for a simple experiment (by combining all cells collected) or six (6) experimental points.
9. CAM-avid cells can continue to be cultured by adding 2-mL CCC medium into each well and transferring plate to incubator.
10. Any unused CAM enzyme working solution can be stored at 2–8 °C for up to 5 days.
11. In case of combining all cells collected, the 6 wells can be sequentially washed each time with the same 3-mL of CCC medium and transferring the wash at the end of each round into the 15 mL tube.
12. Use careful aspiration of the supernatant (by pipetting) to prevent any cell loss.
13. Gating/CTC enumeration can be done directly on BD FACSCalibur™. Our lab typically saves the raw flow cytometry data and analyzes it using FlowJo software.
14. For example, a total of 18 wells can be used to culture cells—among 18 wells, 4 wells without any drug or inhibitor (control); cells in 14 remaining wells can be treated with specific drugs, inhibitors, or any other experimental test the end user might want to try.
15. Refer to Fig. 1 as it demonstrates iCTC counts from 3-day cultures of cells treated with or without two cytotoxic drugs, demonstrating the ability of the Vita-Assay™ to be used in drug sensitivity testing of CTCs.

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