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Abstract

Circulating tumor cell (CTC) as an important component in "liquid biopsy" holds crucial clinical relevance in cancer prognosis, treatment efficiency evaluation, prediction and potentially early detection. Here, we present a Fiber-optic Array Scanning Technology (FAST) that enables antigen-agnostic, size-agnostic detection of CTC. By immunofluorescence staining detection of a combination of a panel of markers, FAST technology can be applied to detect rare CTC in non-small cell lung cancer (NSCLC) setting with high sensitivity and specificity. In combination with Automated Digital Microscopy (ADM) platform, companion markers on CTC such as Vimentin and Programmed death-ligand 1 (PD-L1) can also be analyzed to further characterize these CTCs. FAST data output is also compatible with downstream single cell picking platforms. Single cell can be isolated post ADM confirmation and used for "actionable" genetic mutations analysis.

Key words Circulating tumor cells, Single cell analysis, Non-small cell lung cancer

1 Introduction

"Liquid biopsy" of cancer has been a hot-bed for cancer biomarker research since it provides unparalleled advantage of being minimal-invasive, re-samplable and has the capability of being monitored in real time. Circulating Tumor Cell (CTC) is an important component of "Liquid Biopsy." Since first reported by Ashworth in 1869, there has not been extensive research on these rare cells in cancer patients' peripheral blood till the past decade, primarily due to technical hurdles to enrich these rare cells. Finding these rare cells in peripheral blood, where their number varies from 1 to a few hundreds per milliliter of blood, from millions of leukocytes and billions of erythrocytes, is like finding a "needle in a haystack." Recent technology advances have enabled us to enrich and characterize them primarily based on their unique antigen expression profile [1] or their physical properties (size, deformability, etc.) [2]. However, these technologies have their limitations. Cancer

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cells have been known to undergo Epithelial-Mesenchymal-Transition (EMT) during the metastatic process. During EMT, epithelial cells will shed off epithelial phenotypes, including down-regulation of epithelial antigen—Epithelial cell adhesion molecule (EpCAM) [3], which has been widely adopted as the target molecule for antibody based CTC capture [1]. Meanwhile, other researchers have identified small CTCs that are ~4 μm in diameter [4], and studies on CTC clusters have found un-fixed tumor cells can transverse 5 μm channel by deformation [5]. These evidences indicate that both antigen and size-based methodology can lead to negligence of certain CTC subpopulations. Here, we describe a method that examines all nucleated cells in blood without leaving any subtypes behind using a Fiber-optic Array Scanning Technology (FAST) [6–11], (Fig. 1a). This immunocytochemical assay can rapidly detect rare cells without the need for pre-enrichment, the detected cells are subsequently analyzed in an automated digital microscope (ADM) at high resolution. ADM also allows for multiplex immunocytochemical marker analysis for potential companion diagnostics applications. In addition, the coordinates of identified rare cells can then be utilized for rapid location of the cell on our

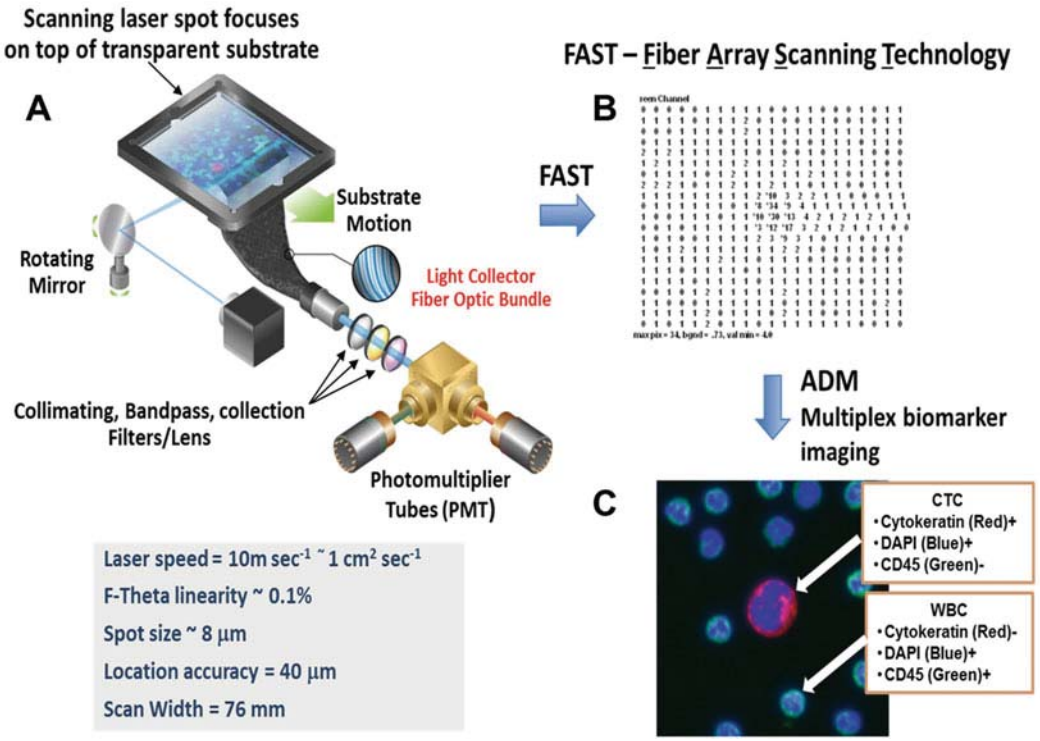

Fig. 1 (**a**) Schematic of Fiber-optic Array Scanning Technology. (**b**) Pixel map representing a target cell detected by FAST. (**c**) After ADM, targets detected by FAST were confirmed as CTC or not CTC by 20× imaging

downstream cell picking platform where automated single cell isolation and downstream genomic analysis takes place.

2 Materials

Prepare all solutions using ultrapure water (prepared by purifying deionized water, to attain a sensitivity of 18 MΩ-cm at 25 °C) and analytical grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise). Diligently follow all waste disposal regulations when disposing waste materials.

2.1 Blood Sample Preparation

1. Ammonium-Chloride-Potassium (ACK) lysing buffer: Prepare 10× ACK lysis buffer as follows: 1.55 M NH_4Cl, 100 mM $KHCO_3$, 1 mM Ethylenediaminetetraacetic acid (EDTA). Weigh 41.45 g of NH_4Cl, 5.00 g of $KHCO_3$, 0.15 g of EDTA, add in 450 mL of water. Mix and adjust pH to 7.4. Make up to 500 mL with water, store at 4 °C. Before use, make 1× ACK lysis buffer. For up to two tubes of blood received (~20 mL), take 10 mL of 10× ACK lysis of buffer, add in 90 mL of water, adjust pH to 7.4, store at room temperature for same-day use.
2. 1× Phosphate-buffered saline (PBS): Prepare 1× PBS by adding 4 L of water to 500 mL of 10X PBS. Adjust pH to 7.4, and then add water to make the volume up to 5 L.
3. Glass slide: Paint with hydrophobic pen (Vector Laboratories) around the edge of the active area on the slide. Pretreat glass slide to remove protective layer as follows: take the 64 cm^2 glass slides (Paul Marienfeld GmbH & Co., KG, Bad Mergentheim), submerge the slides under water in the glass bin for 2 min, then move the glass slides to another glass bin of water for two more min. Then treat the slides with methanol for 15 s to fully remove the protective layer (green) (*see* **Note 1**). Then transfer the slides to a clean bin of water for another 2 min. The slides can then be stored in 1× PBS and ready for use.
4. Prechill acetone (500 mL) at −20 °C.
5. 2% PFA: Dilute 16% Paraformaldehyde (PFA) (Thermo Scientific) 1:7 with 1× PBS.

2.2 Immunofluorescence Staining

1. 20% human serum: Dilute human serum 1:4 with 1× PBS, aliquot into 50 mL cornical tubes and store at −20 °C, prewarm 6 mL of serum per slide at 37 °C water bath before staining steps.
2. 4′,6-diamidino-2-phenylindole (DAPI): Add 1 mL of water into one DAPI vial (Thermo Fisher) to make a 10 mg/mL stock solution. On the day of staining, dilute DAPI stock solution 1:10,000 with 1× PBS for staining.

3. Fluorescence mounting media: Prepare mounting media, 20 mM Tris pH 8.0, 0.5% n-propyl gallate, and 90% glycerol. Weigh 500 mg n-propyl gallate, dissolve in 5 mL water, then add in 2 mL of 1 M Tris buffer, adjust volume to 10 mL, add in 90 mL of glycerol and mix thoroughly. Aliquot into 5 mL per tube and store at −20 °C. Pre-warm the mounting media at 37 °C before use.
4. Primary antibody cocktail: for immunofluorescence staining, mix the antibody cocktail during blocking step. Dilute all antibodies in 20% human serum. An example for the cocktail is as follows: primary antibody: monoclonal mouse IgG1 anti pan-cytokeratin (CK) antibodies for cytokeratin classes 1, 4, 5, 6, 8, 10, 13, 18, and 19 (C2562, Sigma and RCK108, DAKO), monoclonal mouse IgG1 anti-MUC1, EGFR, HER2 and Plastin-3, mouse IgG2a anti-human CD45 antibody (MCA87, AbD Serotec, Raleigh, NC) directly conjugated with Qdot 800 (Invitrogen custom conjugation). In addition, other companion tumor biomarkers can also be added into the primary antibody cocktail such as monoclonal rabbit anti-human Vimentin (EPR3776, Abcam) or monoclonal mouse IgG2b anti-human PD-L1 (329701, BioLegend).
5. Secondary antibody cocktail: biotin-goat anti-mouse IgG1 (A10519, Life technologies), and goat anti-rabbit (A21244, Life Technologies) if labeling Vimentin or anti-mouse IgG2b (A21242, Life Technologies) if labeling PD-L1 antibodies conjugated with Alexa 594.
6. Tertiary antibody: Streptavidin Alexa-555 conjugate (S-32355, Invitrogen).

3 Methods

Carry out all the procedures at room temperature unless otherwise specified.

3.1 Blood Sample Preparation

1. Collect 7.5 mL of blood sample.
2. Add in ACK lysis buffer into the blood at 5:1 ratio (*see* **Note 2**). Rock the sample on a two-axis rocker for 5 min.
3. Centrifuge sample at 300 rcf for 5 min to pellet the remaining leukocytes and rare cells.
4. Confirm that white blood cells (WBCs) have pelleted and aspirate the supernatant.
5. Resuspend cell pellet in 25 mL PBS (*see* **Note 3**).
6. Spin sample at 300 rcf for 5 min.
7. Aspirate the supernatant.

8. Resuspend all cells from each patient in 1 mL PBS (*see* **Note 4**).
9. Count cells on a hemocytometer.
10. Adjust cell suspension to a maximum of 13e6 cells/mL.
11. Remove prepared glass slides from PBS and place in a staining tray. Dry around the active area, carefully, using a Kim-Wipe.
12. Label slides by etching sample ID and date of sample preparation.
13. Add 2 mL of cell suspension per slide (up to 26e6 cells per slide).
14. Incubate for 40 min, in the dark, in a 37 °C incubator for cells to attach (*see* **Note 5**).

3.2 Immunofluorescence Labeling of Tumor Biomarkers

1. Decant cell suspension by tipping slide and pressing edge to a towel.
2. Add 2 mL of 2% PFA in 1× PBS to active plate area (*see* **Note 6**). Incubate for 10 min at room temperature.
3. Decant solution and place plate in a 1× PBS bath for 3 min.
4. Transfer plates to a prechilled acetone bath. Incubate for 5 min at −20 °C (*see* **Note 7**).
5. Transfer slides to a 1× PBS bath for 3 min.
6. Remove from bath, decant excess solution, and wick edges with a towel to remove additional liquid.
7. Add 2 mL of 20% human serum. Distribute evenly onto surface. Incubate at room temp for 30 min.
8. Mix antibodies during incubation.
9. Decant solution from slides and wick off excess.
10. Add 1 mL of primary antibody to patient samples. Incubate 60 min, in the dark, at 37 °C.
11. Decant solution and place in 1× PBS bath at room temperature for 3 min.
12. Transfer to a clean 1× PBS bath at room temperature for 3 min.
13. Decant solution and wick off excess.
14. Dispense 1 mL of secondary antibody and incubate 60 min, in the dark, at 37 °C.
15. Decant solution and place in 1× PBS bath at room temperature for 3 min.
16. Transfer to a clean 1× PBS bath for 3 min.
17. Decant solution and wick off excess.
18. Dispense 1 mL of tertiary antibody and incubate 60 min, in the dark, at 37 °C.
19. Decant solution and place in PBS bath at room temperature for 3 min.

20. Transfer to a clean 1× PBS bath at room temperature for 3 min.
21. Decant solution and wick off excess.
22. Dispense 2 mL of pre-diluted DAPI and incubate in the dark, at room temperature, for 10 min.
23. Decant solution and place in 1× PBS bath at room temperature for 3 min.
24. Dip plate into ddH_2O, briefly (~10 s).
25. Coverslip the slide (*see* **Note 8**).

3.3 Fiber-Optic Array Scanning Technology for Candidate CTC Coordinate Identification

1. Use nail polish to seal the edges of the coverslip on the FAST slide.
2. Place four fiduciary markers on the top of the nail polish, aligned with the printed fiduciary mark along the edge of the cover slip, on the four corners of the slide.
3. Turn on the FAST scanner and allow the scanner to warm up for 15 min.
4. Open software on the FAST computer.
5. Place the slide onto the FAST scanner.
6. Initiate the scan in the FAST software.
7. After the scanning is done (about 1 min per slide up to 26e6 cells), save the file onto the server. An example of the generated pixel map can be found in Fig. 1b.
8. Found CTC candidates using "RawV2" software. Use the "Find Cells" function to find coordinates that meet the setting criteria of potential CTCs.

3.4 Automated Digital Microscope (ADM) Imaging for CTC Confirmation

1. Turn on microscope and open the software "RawV2" for ADM.
2. Open the FAST file that was saved on the server on the last step.
3. Calibrate the microscope to read the coordinates of potential targets for automated imaging: Register the fiduciary marks on the FAST scan image, zoom in on the scanned image to locate the fiduciary marks. In the "calibrate" mode, click on the center of each fiduciary mark in the sequence of "upper left," "lower left," and "upper right" corners.
4. Load the slide onto the microscope stage. Find centers of the fiduciary marks under 20× bright field, in the same sequence as in finding the marks on the FAST scanned image. Once the center of each mark is located under the microscope, use "Get x,y" function to get the coordinates of each fiduciary mark (Fig. 2). And then click "Apply."

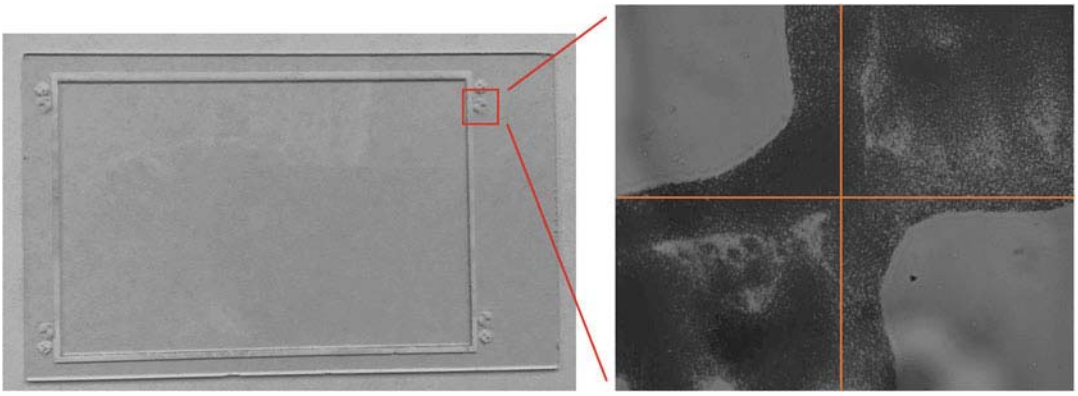

Fig. 2 Fiduciary markers (in *red box*) are placed onto the FAST slide and used for calibration of coordinates on ADM and cell picking platform

5. In the Camera tab, adjust exposure time for each channel (*see* **Note 9**).
6. Click "Capture," to capture a 3-channel image. Confirm the exposure time for DAPI, TRITC, and Q-dot 800 is appropriate.
7. Use "autofocus" function to determine the off-set between the center of true focus and the focus the software determines. Enter that value into the software to off-set the error in Z-axis.
8. Click "Start" to sequentially image all the potential target coordinates on the slide.
9. Review all images to determine which ones are "true positive" CTCs, by the criteria of cytokeratin (or tumor markers such as MUC1, HER2, EGFR, or plastin-3) positive intact cells with DAPI stained nucleus inside cytoplasm, at least 4 μm in size and negative for CD45 staining (Figs. 1c and 3a).
10. If additional biomarkers are stained on CTC for grading, such as Vimentin or PD-L1, all "true positives" need to be reimaged in the other channels. This can be done by labeling all "true positives" for retake. Then choose to take multiple images in Alexa-fluor 647, Alexa-fluor 700, Alex-fluor 750 or Qdot 705 channel in addition to the three channels that are used for CTC identification (DAPI, TRITC and Qdot 800). (Fig. 3b) (*see* **Note 10**).

3.5 Single Cell Isolation and Sanger Sequencing of CTCs Detected by FAST

1. Generate the pick target transfer list using the "Compute Statistics" function in the RawV2 software. Format the X.Y coordinates of the detected CTCs into an Excel worksheet and save the formatted pick list table into "comma separated values" (.csv) format.
2. Submerge the sample slide into 1× PBS for 30 min until the coverslip on the slide completely detaches (*see* **Note 11**).

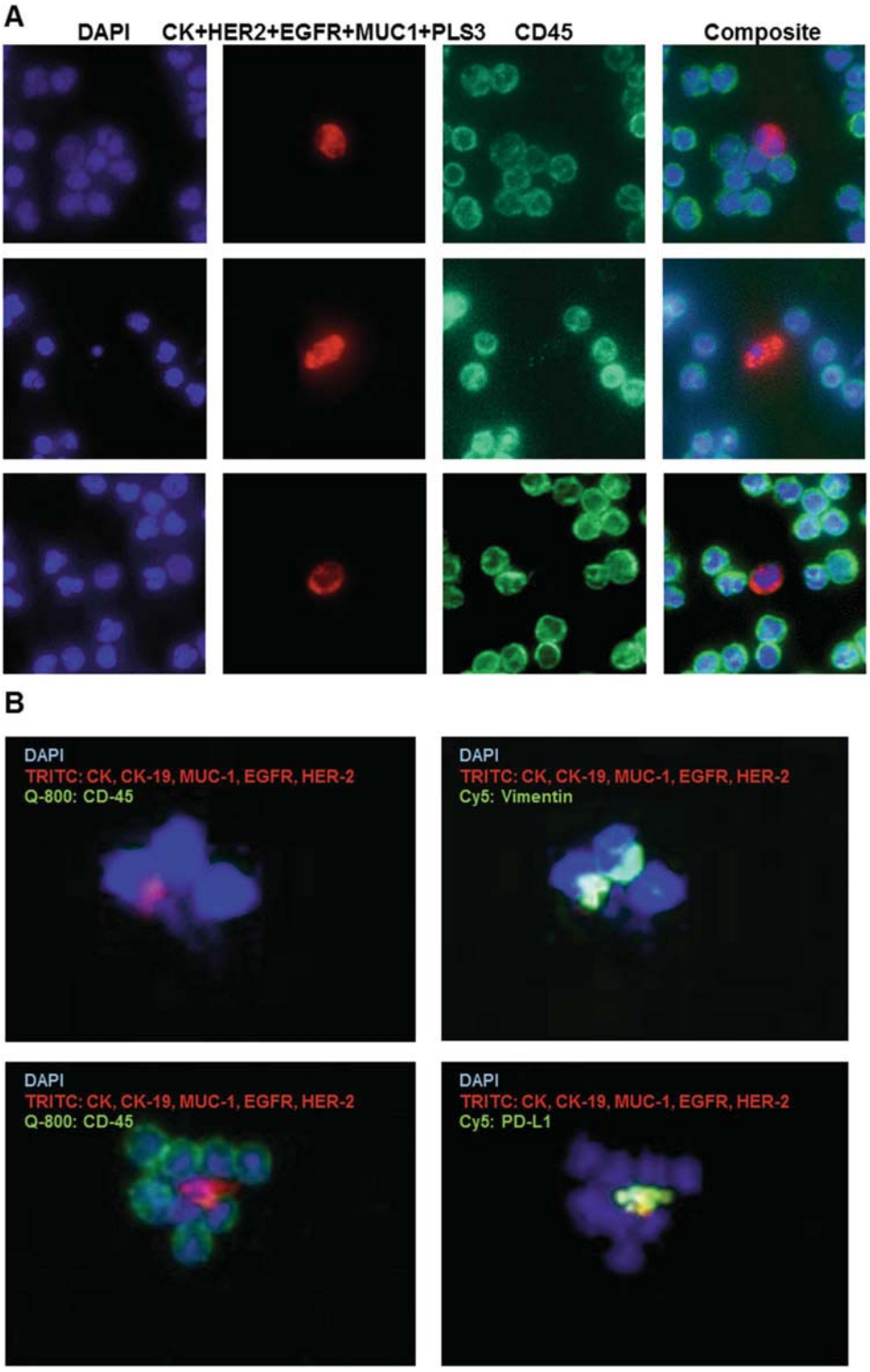

Fig. 3 (**a**) CTC can be identified as DAPI+, tumor marker+, CD45– cells with intact morphology and size > 4 μm. (**b**) Companion biomarkers can be analyzed on CTCs such as Vimentin and PD-L1

3. Place the uncoverslipped slide in a customized plate holder and transfer it to the ALS CellCelector platform.
4. Apply 1.5 mL of 1× PBS onto the active area of the slide using a 1 mL pipette to hydrate the sample.
5. Open the CellCelector software with the built-in function interface for pick list coordinates transfer. Import the ".csv" transfer list file.
6. Calibrate the coordinates four fiduciary marks by aligning them in the center of the field of view under 20× bright field. Click on the center of each mark to get the coordinates.
7. Click on each pick target to make sure that after calibration, they are centered in the field of view using both 20× bright field and 20× TRITC image settings. If not, use the "calibrate" function to re-center the target.
8. Place a 96-well PCR plate on the picking platform with 5 μL of cell extraction buffer preloaded into each well.
9. Calibrate the pick-up position and deposit height using the built-in functions in the software.
10. Mount a 30 μm diameter capillary onto the CellCelector. Sterilize the picking capillary by aspirating and dispensing 70% ethanol with the capillary.
11. Initiate the picking program (*see* **Note 12**).
12. Perform Whole-Genome Amplification (WGA) on the single cell deposited.
13. Examine the WGA product by electrophoresis (Fig. 4d) (*see* **Note 13**).
14. Use the WGA product for targeted PCR for genes of interest.
15. Confirm gene amplicon is again by electrophoresis (Fig. 4e).
16. If desired amplification is confirmed, clean up the product.
17. Measure the concentration of the purified product.
18. Adjust the concentration of the amplicon to 10 ng/uL. Prepare the sequencing reaction according to Table 1.
19. Sequence the target amplicon. An example of the result can be found in Fig. 4f.

4 Notes

1. Check if the protective layer (green) is fully removed from the slide, and if not, treat with methanol for an additional 10 s.
2. Use 1× ACK buffer to rinse the pipette used for transferring the blood and the collection tube(s) to ensure all cells were collected into the lysis tube.

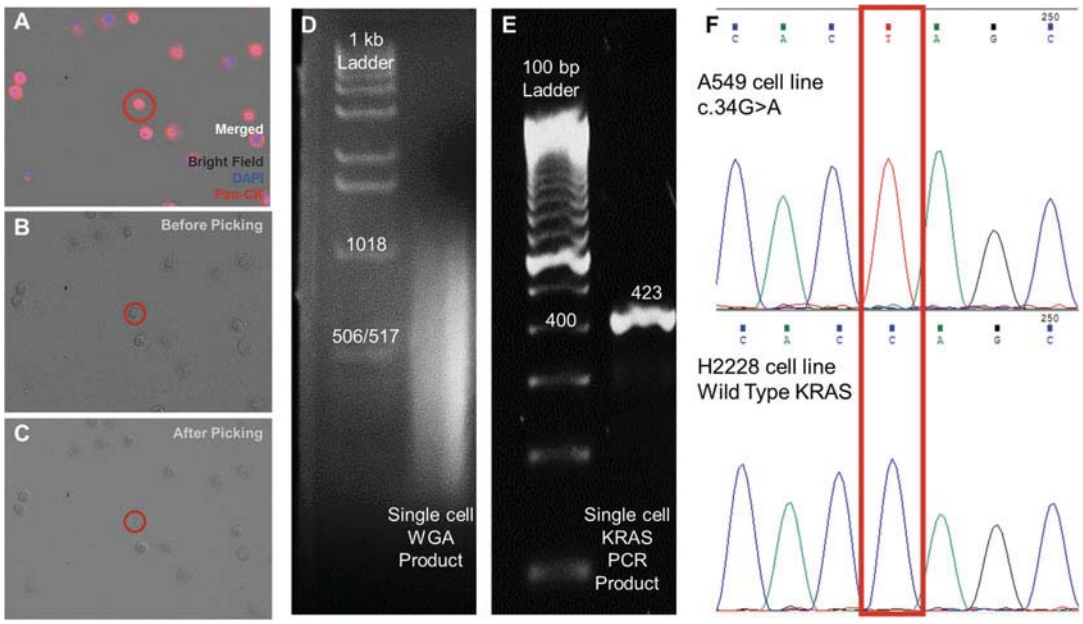

Fig. 4 (**a**) Merged image of immunofluorescently stained human A549 cell line to be picked. (**b**) A549 target cell in 20× bright field before picking. (**c**) A549 target cell in 20× after picking. (**d**) After whole genome amplification, the product is analyzed by electrophoresis. (**e**) KRAS gene is amplified by PCR, and the amplicon is analyzed by electrophoresis. (**f**) Sanger sequencing results indicate KRAS point mutation c.34 G>A detected in positive control cell line A549 but not in H2228 cell line as expected

Table 1
Sequencing reaction preparation

Reagents	Volume
PCR product (10 ng/μL)	10 μL
Custom primer (forward or reverse)	2 μL
Total volume	12 μL

3. Flick the tube gently until the entire pellet is resuspended into 1× PBS.
4. If more than one tube is used for blood lysis, use 1 mL of 1× PBS to first resuspend the pellet in one tube and then transfer the cell suspension (1 mL) to the next tube to resuspend the next pellet in the same 1 mL of 1× PBS; thus, all PBMCs from the same patient will be resuspended in the same 1 mL of 1× PBS.
5. Check the plate for cell suspension distribution after 3 min incubation. Make sure the cell suspension is dispensed evenly on the plate, and if not, re-level the shelf in the incubator until

the cell suspension can be distributed well on the plate without areas drying.

6. Gently add the 2% PFA onto the plate using a 1000 μL pipette. Adding the 2% PFA from too high above the plate may result in cells lifting up from the plate. Carefully roll plate to distribute 2% PFA evenly, and use a pipette tip to push solution all the way to the edges and corners.
7. Five slides can be treated with acetone in each glass tray. If more than five slides are being treated, prechilled acetone can be used for up to three batches of slides sequentially. After acetone treatment, slides can be air-dried and stored at −80 °C. If slides are stored, re-treat slide with prechilled acetone for 5 min when bringing out the slides from −80 °C before proceeding to **step 5** (Subheading 3.2).
8. Coverslip the slide with FluorSave mounting medium (345789, Millipore) if the endpoint is CTC enumeration. Coverslip the slide with the house-made fluorescence mounting media (*see* Subheading 2) if the endpoints is to uncoverslip and pick single cells for Sanger Sequencing.
9. Adjust the exposure time for each channel as follows: adjust DAPI exposure so that about 30% of leukocyte nuclei are partially saturated. Switch to Q-dot 800 channel (CD45) and adjust exposure so that 10% of leukocytes show saturated signal around their circumference and the signal is also visible in the internal region of the cell (i.e., where view angle is perpendicular to the cell membrane).
10. For quantification of companion biomarker, a moderate expressor for each sample was used as positive control, and leukocytes from the same sample were used as negative control. The expression level was determined by averaging the intensity of the pixels in the region where the marker protein was expressed. Then CTCs with an expression level between the 16th and 68th quantiles of the positive control distribution were scored 2, while the CTCs expressing higher levels were scored 3. CTCs expression level lower than the 68th quantile was scored as 0. The percent population was scored linearly, as less than 10% of population of CTCs expressing the marker was scored as 0, and 90% to 100% of the CTC population expressing the marker was scored as 10.
11. If the coverslip does not fully detach after PBS treatment, gently lift up the coverslip using forceps. Try avoiding sliding of the coverslip on the sample while lifting.
12. Set the picking parameters in the CellCelector program as follows: Pre-pick buffer volume: 1.5 μL (PCR grade water in buffer tank); aspiration volume: 1.5 μL; aspiration speed: 1%; scraping distance: 40 μm; scraping speed 2%. After each pick,

check the pre- and post-pick image to ensure the cell was successfully picked without picking up neighboring cells (Fig. 4a–c).

13. The majority of the WGA product should be at a length of between 200 bp and 1 kb.

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