Motion microscopy for label-free detection of circulating breast tumor cells

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ABSTRACT

Circulating tumor cells (CTCs) are cancer cells that have been shed from a primary tumor and circulate in the bloodstream during progression of cancer. They may thus serve as circulating biomarkers that can predict, diagnose and guide therapy. Moreover, phenotypic and genotypic analysis of CTCs can facilitate prospective assessment of mutations and enable personalized treatment. A number of methodologies based on biological and physical differences between circulating tumor and non-tumor cells have been developed over the past few years. However, these methods did not have sufficient sensitivity or specificity. In this work, a remote analysis protocol was designed using motion microscopy that amplifies cellular micro motions in a captured video by re-rendering small motions to generate extreme magnified visuals to detect dynamic motions that are not otherwise visible by naked eye. Intriguingly, motion microscopy demonstrated fluctuations around breast tumor cells, which we referred to herein as cellular trail. Phenomena of cellular trail mostly emerged between 0.5 and 1.5 Hz on amplified video images. Interestingly, cellular trails were associated with cell surface proteins and flow rates rather than mitochondrial activity. Moreover, cellular trails were present only around circulating tumor cells from individuals with breast cancer under conditions of 20–30 μm/s and 0.5–1.5 Hz. Thus, motion microscopy based CTC detection method can offer a valuable supplementary diagnostic tool for assessment of drug efficacy and identifying physical characteristics of tumor cells for further research.

1. Introduction

The metastatic process is complex with CTCs ultimately seeding in a distant site where they adapt to a new environment by neoangiogenesis (Lambert et al., 2017). CTCs in most individuals with cancer are lower than 10 to 100 cells per milliliter of blood (He et al., 2008). CTCs have been identified in many cancers, including breast, lung, prostate, pancreas, stomach, and colon (Bidard et al., 2018; Dong et al., 2002; He et al., 2008; Tsai et al., 2016). Several techniques have been used and applied for CTC detection (Supplementary Table 1). These techniques are composed of density-based separation, microfilters, microfluidic sorting, immunoaffinity, or combination of these methods. Traditionally, density gradient centrifugation is employed to enrich the mononucleocyte fraction, which includes CTCs due to their similar buoyant density. However this method can be time consuming (Rosenberg et al., 2002; Campton et al., 2015). On the other hand, CTC isolation using microfilters has been demonstrated to be efficient, by exploiting the size of CTCs which are significantly larger than circulating blood cells. However, the track etching of microfilters often results in fusion of two or more pores, resulting in lower CTC capture efficiency.
A method is expensive owing to cost of antibodies conjugated to magnetic beads with wide ranging yield (9–90%) due to variable expression of surface markers. Recently, combinations of technologies have been developed to overcome various shortcomings of these detection methods (Yoon et al., 2016; Hosokawa et al., 2010). Additionally, CTC surface epithelial marker enrichment such as EpCAM, HER2, EGFR can be used (Sajay et al., 2014).

The motion microscopy is a computational tool that quantifies micro motions from videos by generating a new image whereby the motions are magnified enough to be visualized by human eye (Adiv, 1985; Hurlbut and Jaffey, 2015; Sellen et al., 2015; Wadhwa et al., 2017). Through this methodology, otherwise invisible micro-movements are recorded which can subsequently be visualized by pixel camera. Therefore, more pixels covering the object of interest would yield better signals for extraction. For every pixel at location \((x, y)\), time \(t\), scale \(r\), and orientation \(\theta\), spatial local phase information was combined in different sub-band of frames using the least squares objective function (Wadhwa et al., 2017),

\[
\arg \min_{\phi_{i,R}, \psi_{i,R}} \sum_{i,R} \left( \frac{\partial \phi_{i,R}}{\partial x} \frac{\partial \psi_{i,R}}{\partial y} - (u, v) - \Delta \phi_{i,R} \right)^2.
\]

Arguments have been suppressed for readability: \(A_{i,R}(x, y, t)\) and \(\phi_{i,R}(x, y, t)\) are the spatial local amplitude and phase of a steerable pyramid representation of the image, and \(u(x, y, t)\) and \(v(x, y, t)\) are the horizontal and vertical motions at every pixel (Wadhwa et al., 2017).

The original purpose of motion microscopy was to measure vibrations of building structures or earthquakes, and the detectable movement was 0.3 nm–100 nm in macro-spheres (Adiv, 1985; Wadhwa et al., 2017). However, this has not been described in micro-spheres, such as in areas of cellular properties. Applying the image from a microscope, we expected to be able to detect vibrations between 1 nm and 0.003 nm using a motion microscope and examined whether it could be applied to detect fluctuations of tumor cells. We therefore hypothesized that motion microscopy can be used to detect tumor cells. As CTCs of breast cancer have been studied extensively and can be a promising potential for liquid biopsy (Bidard et al., 2018), we analyzed wavelength profiles in breast cancer cells using motion microscopy.

2. Materials and methods

2.1. Cell lines and culture

MCF-7 (HTB-22, ATCC, USA), MDA-MB-231 (HTB-26, ATCC, USA), and SK-BR-3 (HTB-30, ATCC, USA) were maintained in Dulbecco’s MEM (11885, Gibco, USA) with 10% fetal bovine serum (10270010, Gibco, USA). All cultures were maintained at 37 °C under an atmosphere of 95% \(\text{O}_2\) and 5% \(\text{CO}_2\). Before each experiment, cells were detached from the surface of culture flasks by 0.05% trypsin (15400054, Gibco, USA). The resulting cell suspensions were then centrifuged at 118 \(\times g\) and resuspended with 1 × phosphate-buffered saline buffer (PBS; 10010023, Gibco, USA). Cells were washed by spinning down at 118 \(\times g\) and resuspending into a new PBS solution. To prepare leukocytes, human whole blood (HUMANWBB2, BIOIVT, USA) was lysed with red blood cell lysis buffer (ab294733, abcam, USA) in a volume ratio of 1:10. The mixture solution was incubated for 10 min at room temperature and then centrifuged at 800 \(\times g\). The supernatant was removed and the cell pellet was resuspended using PBS. Peripheral blood mononuclear cells (PBMCs) were isolated from individuals with breast cancer (HMPBMC, Santracruz Biotechnology, USA).

2.2. Experimental procedures of microfluidic device and motion microscopy

Microfluidic devices (Polydimethylsiloxane chip, Microfit, South Korea) were placed on the stage of an inverted microscope and the fluid flow was controlled by individual syringe pumps (BS-9000-12, Braintrace scientific, USA). The microfluidic device and syringe pumps were connected by polythene tubing (PE10, Braintrace scientific, USA) with an inner diameter of 0.28 mm. Prior to each experiment, isopropanol (W292907, Sigma-Aldrich, USA) was flushed through the whole microfluidic device to remove all of the air bubbles in the channel followed by 1 X PBS (10010023, Gibco, USA) wash for 30 min. Breast tumor cells, leukocytes, PBMC or mixture were then introduced to the device at a flow rate of 20–30 \(\mu m/s\) and video files were recorded through the inverted microscope at 1200 \(\times\) 512 pixels and 500 frames per second. The recorded videos were uploaded to https://lambda.qrlab.com/site/ and the magnification type was selected in colour mode, with amplification ratio of 20, and wavelength was selected from 0.5 Hz to 5 Hz in conversion condition.

2.3. Measurement of ATP

Intracellular ATP levels were measured using a colorimetric/fluorometric assay kit (ab83355, abcam, USA). Briefly, 10\(^6\) cells were treated with 1 mM Na\(_3\)V or 1 mM PBS for 5 h, then lysed in the ATP assay buffer. Cell lysates were centrifuged at 15,000 \(\times g\) for 2 min and the supernatant was added to a 96-well plate followed by addition of 50 \(\mu l\) of the Reaction Mix to each well. The absorbance was read at 550 nm using a microplate reader (SYNERGY H1, BioTek, USA). ATP content was calculated based on a simultaneously generated standard curve.

2.4. Analysis of mRNA using real-time PCR

The extracted mRNA were determined using ERBB2 (Hs01001580_m1), ERBB3 (Hs00176358_m1), IGF1R (Hs00609566_m1), CTNNAL1 (Hs00426996_m1), CTNNB1 (Hs00355045_m1), CTNND1 (Hs00931670_m1), ITGA2 (Hs00158127_m1), ITGB1 (Hs01127536_m1), CD9 (Hs01124026_m1), CD97 (Hs0173542_m1), GNB1 (Hs00297997_m1), GNB2L1 (Hs00272002_m1), KRT8 (Hs01595539_g1), KRT18 (Hs02827483_g1), KRT19 (Hs00761767_s1), TUBA1B (Hs00744842_sH), TUBB (Hs00742828_s1), ACTG1 (Hs03044422_g1), ACTG1 (Hs01109515_m1), ACTA2 (Hs00426835_g1), MYH9 (Hs00159522_m1), MYH10 (Hs00992055_m1), MYO1B (Hs00362654_m1), MYO1C (Hs00300761_m1) primer/probe set from Life Technology. Real-time PCR was performed with TaqMan universal PCR Master Mix on an ABI Real time PCR System 7000 (Applied Biosystems, USA). PCR conditions were 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. For each experimental sample, the relative abundance value was normalized to the value derived from TBP (Hs00427620_m1, Life Technology) as housekeeping control gene. Relative mRNA levels were quantified using the comparative 2^-\Delta\Delta C_t method.

2.5. CRISPR-mediated gene deletion

Clustered regularly interspaced short palindromic repeats (CRISPR) transfection of ACTG1, CTNNAL1, GNB1, or ERBB2 in MCF-7, MDA-MB-231, or SK-BR-3 was performed using a kit from Santa Cruz (sc-395739, Santa cruz Biotechnology, USA). Briefly, in six-well culture plates, 10^6...
cells were plated and exposed to the ACTG1-CRISPR plasmid (sc-400006, Santa Cruz Biotechnology, USA), CTNND1-CRISPR plasmid (sc-400943, Santa Cruz Biotechnology, USA), GNB1-CRISPR plasmid (sc-401901, Santa Cruz Biotechnology, USA), ERBB2-CRISPR plasmid (sc-420218, Santa Cruz Biotechnology, USA) or negative control-CRISPR plasmid (sc-418922, Santa Cruz Biotechnology, USA) solution for 8 h at 37 °C in a CO2 incubator. Then, the media was changed to Dulbecco’s MEM with 10 % fetal bovine serum and incubated for another 18 h. The ACTG1, CTNND1, GNB1, ERBB2 expression were determined using western blotting.

2.6. Western blot

Briefly, MCF-7, MDA-MB-231, SK-BR-3 cells, or leukocytes were homogenized in ice-cold lysis buffer. After centrifugation at 5,000 g for 20 min, protein content of the supernatant was quantified using a Bradford protein assay. Samples were diluted, boiled with sample loading dye, and 100 mg were loaded in SDS-PAGE (4561033EDU, Bio-Rad). After blotting, membranes were blocked in 5 % skim milk (70166, Sigma-Aldrich) in PBS containing 0.1 % Tween-20 (P1379, Sigma-Aldrich). Membranes were incubated with antisera directed against ACTG1 (1:1000; sc-65638, Santa Cruz Biotechnology, USA), CTNND1 (1:1000; sc-23873, Santa Cruz Biotechnology, USA), GNB1 (1:1000; ab137635, abcam, USA), and ERBB2 (1:1000; ab134182, abcam, USA), and then with secondary antibodies (mouse-specific HRP-conjugated antibody or rabbit-specific HRP-conjugated antibody). Bands were visualized using ECL (32106, Thermo Scientific) detection kit and quantified by densitometry. Blots were stripped and re-exposed to detect TBP (1:1000; ab28175, abcam, USA) as housekeeping protein.

2.7. Fluorescence microscope

Briefly, transfection of pRSET-BFP (V35420, Invitrogen, USA) in MCF-7, MDA-MB-231 or SK-BR-3 cells was performed using a kit from Santa Cruz (sc-395739, Santa Cruz Biotechnology, USA). PBMCs were placed in 10% formalin for 3 h and incubated with antisera against FITC-conjugated ERBB2 (1:400; ab31891, abcam, USA). After washing with PBS, cells were visualized using Zeiss LSM 510 confocal microscope (Carl Zeiss, German).

2.8. Statistical analysis

Values are means ± SE. The significance of differences was determined by a two-way analysis of variance (ANOVA), or a one-way ANOVA followed by a Bonferroni post-hoc analysis where appropriate. Differences were considered significant when P < 0.05.

3. Results and discussion

Detection of CTCs is an important technique for the initial diagnosis of cancer metastasis and for monitoring the response of various cancer therapies (Bidard et al., 2018; He et al., 2008; Tsai et al., 2016; ). A number of different techniques have been developed for CTC detection to date. However, many methods have pitfalls with suboptimal efficiencies and specificities. For example, detecting CTCs based on size differences is insufficient due to lack of ideal biomaterial for selection based on cell size differential (Coumans et al., 2013). Monoclonal antibodies to detect surface markers of CTCs are also not sufficient due to their high variation in gene expression (Sieuwerts et al., 2009). We describe here a novel method of remote analysis using motion microscopy that can amplify to visualize extreme micro motions of tumor cells from pixels of the recorded videos.

3.1. Contact free analysis of oscillating breast tumor cells using motion microscopy

To overcome the difficulty of focusing on continuous recordings of multiple moving cells, we had cells rolling on the surface using microfluidics. As MCF-7 cells are known to have acoustic physical properties, we initially used this cell line for motion microscopy (Li et al., 2015). MCF-7 cells were subjected to flow on a polydimethylsiloxane based microfluidic channel at a flow rate of 20–30 μm/s and then recorded at 1200 × 512 pixels at 500 frames per second. Micro movements of MCF-7 cells were amplified by a motion microscope and the detailed settings were 20 times in amplification rate, colour mode in magnification type, and 0.5 Hz in wavelength (Fig. 1A and B). Intriguingly, fluctuations were evident around MCF-7 cells, which we referred to herein as cellular trail in amplified movies (Fig. 1C).

3.2. Various oscillating shapes of breast tumor cells according to wavelengths

Since diversiform vibrations of cancer cells had been reported from 0.5 Hz to 5 Hz (Nelson et al., 2017), the invisible movements of MCF-7 cells were amplified using a motion microscope from 0.5 Hz to 5 Hz. Here, 1 Hz is 60 invisible repetitive movements for 60 s. Intriguingly, cellular trails began to appear at 0.1 Hz and emerged with clarity from 0.5 to 1.5 Hz. These cellular trails began to disappear at 1.5 Hz and eventually became difficult to detect from 2 to 5 Hz (Fig. 2A and Supplemental movie 1-7). In order to investigate whether cellular trails were detectable in different breast tumor cells, the invisible movements of MDA-MB-231 or SK-BR-3 cells were amplified using a motion microscope from 0.5 Hz to 5 Hz. Similar cellular trails of MDA-MB-231 or SK-BR-3 were detected from 0.5 to 1.5 Hz, and disappeared from 2 Hz to 5 Hz (Fig. 2B and C). According to these results, the level of intensity and wavelengths of cellular trails were similar between different types of breast tumor cells (Fig. 2D).

Supplementary video related to this article can be found at http://doi.org/10.1016/j.bios.2020.112131

3.3. Cellular trails are not associated with mitochondrial activity

Since the origin of fluctuation of cancer cells were not known, we first postulated the mitochondria, as they have been reported to cause vibrations in tumor cells (Nelson et al., 2017). To test this hypothesis, MCF-7, MDA-MB-231, and SK-BR-3 cells were treated with 1 mM NaN3, a cytochrome oxidase inhibitor, to block mitochondrial activity, and invisible movements were amplified using the motion microscope. Fig. 3A demonstrates that 1 mM NaN3 effectively inhibited mitochondrial activity as assessed by significant suppression of intracellular ATP content of all tumor cells. Interestingly, despite suppression of the mitochondrial activity, the intensity of cellular trails was not changed (Fig. 3B and C). These results suggest to our surprise that the appearance of cellular trails is not likely dependent on intracellular ATP levels.

3.4. Role of cell surface proteins on cellular trails

We next examined other potential parameters that may affect changes in cellular trails in motion microscopy. One such factor is a frictional force between surfaces of breast tumor cells (Sandnes et al., 2011). Among various types of frictional force, fluid friction was considered as it is mainly affected by the surface texture and the amount of force impelling them together. Therefore, cell surface proteins that could influence fluid friction were investigated. Tyrosine kinases (ERBB2, ERBB3, IGF1R), cell adhesion markers (CTNNAL1, CTNNAL1, CTNND1, CTNNAL1, ITGB1, ITGB1, CD9), GPCRs (CD97, GNAI3, GNB1, GNB2L1), structural proteins (KRT8, KRT18, KRT19, TUBA1B, TUBB, ACTG1, ACTC1, ACTA2, MYH9, MYH10, MYO1B, MYO1C) are common cell surface proteins of MCF-7, SK-BR-3 and MDA-MB-231 which were
examined (Ziegler et al., 2014). Quantitative real time-PCR showed high levels of ACTG1, CTNND1, and GNB1 (Fig. 4A). ERBB2 is a known specific marker of breast tumor cells (Harari and Yarden, 2000). While this marker is expressed in relative small quantities, we felt it was necessary to assess the role of this cell surface protein in motion microscopy. To elucidate the relationship between cell surface proteins and cellular trail, ACTG1, CTNND1, GNB1 or ERBB2 were deleted using transfection of CRISPR-Cas9 plasmid (Fig. 4B). Remarkably, deletion of ACTG1 or CTNND1 significantly reduced the intensity of cellular trails in motion microscopy. However, GNB1 or ERBB2 appeared not to be required for cellular trails of breast tumor cells (Fig. 4C). In particular, ERBB2, a selective marker for breast cancer, appeared not to be required for the fluctuation, implying that the motion microscope method likely does not overlap with biomarker detection methods using antibodies. The efficiency of ACTG1, CTNND1, GNB1 or ERBB2 knockdown was confirmed using Western blot (Fig. 4D-F). Also, various cellular trails

Fig. 1. Motion microscope contact-free detection system to measure cellular vibration. (A) Schemata of the experimental setup of microfluidic device and motion microscope. MCF-7 breast tumor cells were subjected to the microfluidic device at a flow rate of 20–30 μm/s and video recording files were obtained from the microscope at 1200 × 512 pixels at 500 frames per second. Motion microscope amplified micro motions by video using spatial local phase. (B) Photographs of microfluidic device on the inverted microscope. (C) Cellular motions were amplified 20 times in color mode to obtain frames of magnified images. The motion microscope revealed modal shapes of MCF-7 (arrow head) on converted video. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Fig. 2. Motion magnified video of breast tumor cells revealed different modal shapes at various wavelengths. The images of micro motions of MCF-7 (A), MB-231 (B), SK-BR-3 (C) were converted by a motion microscope at 0.5 Hz–1 Hz. All three tumor cells showed distinct cellular trails (arrow heads) between 0.5 Hz and 1.5 Hz. (D) Intensity levels of cellular trails in motion magnified videos between 0.5 Hz and 5 Hz. Results are the means ± SE of 6 experiments in each group. *Significantly different from motion magnified videos at 0.1 Hz–0.5 Hz, P < 0.05.
were observed with PBS, Krebs solution, saline, RPMI 1640, human plasma or high concentrations of FBS (Supplementary Figs. 1A–C). Of note, cellular trails are dependent on the state of the cells and are not always present at a constant intensity.

3.5. Alteration of flow rates is associated with cellular trails

Flow rate can affect the frictional force of the cell surface (Byun et al., 2013). Therefore, to assess its effect, changes in flow rates were given at an amplification wavelength from 0.5 to 1.5 Hz. In an environment with an elevated flow rate of three times basal, from 70 to 90 μm/s, the intensity of cellular trails were augmented two-fold (Fig. 5A). Although...
The flow rate could be further increased, it was difficult to detect each cell in moving images at above 100 μm/s. On the other hand, when the flow velocity was reduced to zero, cellular trails of the breast cancer cells disappeared (Fig. 5A). We also tested leukocytes which expressed a small amount of ACTG1 among proteins that can affect the cellular trail. However, there was no detectable cellular trail at a flow rate of 20–30 μm/s (Fig. 5B). Unlike tumor cells, cellular trails were only faintly observed in leukocytes at a flow rate of 70–90 μm/s and were not detectable at a flow rate of 20–30 μm/s (Fig. 5A and B). This allows tumor cells to be clearly distinguished from leukocytes in the blood of patients with breast cancer under the same conditions of motion microscope.

3.6. Motion microscope increases sensitivity of detecting breast cancer cells

We next assessed a mixture of leukocytes with breast tumor cells under conditions of 20–30 μm/s and 0.5–1.5 Hz and found that cellular trails can be clearly distinguished between tumor cells and leukocytes (Fig. 6A). Under these ranges of velocity and wavelength, detection of cancer cells was also visible in peripheral blood mononuclear cells (PBMC) of patients with breast cancer (Fig. 6B and Supplementary Table 2). Cellular trails were detected at visible light (Fig. 6C) and tumor cells were confirmed using fluorescence microscopy (Fig. 6A and B). Using cell size difference, breast tumor cells were isolated using Screen Cell cyto, and this detection method was compared to that of motion microscopy. Total numbers of breast tumor cells were counted manually using fluorescence microscopy. The conventional size differential detection method yielded a sensitivity of 68%–73%, whereas motion microscopy method detected tumor cells with a sensitivity of 93% (Fig. 6D). Moreover, the motion microscopy method yielded not only a higher detection rate but was also more consistent, while the size-differential method had a large variation in results (Fig. 6E).

Overall, cell adhesion and structural proteins such as CTNNBD1 and ACTG1 appeared to be essential in affecting the cellular trails and tumor cells were clearly distinguishable from leukocytes using the motion microscope under condition of 20–30 μm/s and 0.5–1.5 Hz (Fig. 6F). In summary, we show that the motion microscope can detect tumor cells at more consistent and higher rate than conventional methods such as using biomarkers via antibodies or other physical characteristics of tumor cells. This can potentially facilitate early diagnosis by detecting parameters that may precede the current conventional markers. It may also provide complementary supplemental information to further enhance sensitivity and specificity of breast cancer detection. Moreover,
recurrence after treatment can be detected which can serve to survey response to further treatment. Together, we offer a novel computational tool for detection of tumor cells which may be used for assessment of drug efficacy and for physical characteristics of tumor cells for further research.

4. Conclusion

Micro motions of cells can reveal important dynamic changes under various biological conditions. Motion microscopy facilitates visualization of tumor cell micro movements and formation of cellular trails using a digital camera. We show in this study that cellular trails were observed specifically in tumor cells and CTCs were detected from human blood samples with a consistent detection rate.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: I have numerous grants from Korea government and Ewha Womans University.

CRediT authorship contribution statement


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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bios.2020.112131.

References


