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BRIEF REPORT

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High counting of circulating tumor cells in blood is not directly related to metastasis

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Abstract

Circulating tumor cells (CTCs) in blood flow have been believed as an essential biomarker of cancer. The technologies of in vitro and in vivo CTC enrichment and detection suggest although CTCs might play a role of "seed" in metastasis, only the minority of CTCs, probably in the form of CTC clusters, hold the potential to develop a tumor in organs. The detected amount of CTCs might be solely an indicator of tumor burden. To provide new insights into this argument, we take advantage of a safe drug to tune the pacemaker activity of a mouse tumor model to increase the heart rate for a period of time every day during the tumor development. We detect the CTCs in vivo by fast line scanning of a confocal microscope when the heart rate returns to the baseline and find the average CTC amount is significantly elevated in the drug-treated group but the metastases are even less than that of control. Our results imply the detected CTC counts in blood might not be directly related to metastasis.

KEYWORDS
circulating tumor cells, heart rate, in vivo detection, metastasis

1 | INTRODUCTION

Cancer metastasis is the main reason for death induced by cancer and makes cancer therapy extremely difficult and complicated. The migration mechanism of tumor cells remains unclear, which is believed involved with the detachment of tumor cells from the primary tumor, their entry to the circulating systems to form the so-called circulating tumor cells (CTCs), the long-term survival of CTCs in circulating systems, and final settlement in organs with a soak of tumor cells and angiogenesis [1–4]. In this theory, CTCs play an essential role of the "seed" in metastasis. In circulating systems, the CTCs that survive from the immune attack and mechanical stress of blood might take a small chance to migrate through the vascular epithelial to organs. Recently, CTCs in blood have been working as an important biomarker of tumor generation and prognosis [3, 5–7]. A series of clinical researches reported that the amount of CTCs in blood is highly related with the survival rate of cancer patients [8–10]. Although more CTCs in blood theoretically increase the chance that CTCs stay in the vascular wall and invade organs, some recent researches have indicated that only a small part of CTCs, like CTC clusters, hold the potential to defend the immune attack, construct a niche, and develop into a tumor. The major part of CTCs can hardly survive, migrate, and engage in a right niche [2, 3]. Whether the detected increase of CTC amount is solely a biomarker of tumor burden or a key to metastasis is still a controversial issue.

The CTCs in blood are quite rare, at the level of 1 CTC/1 ml blood, which is, therefore, greatly challenging for detection. There are mainly two types of technologies for CTC harvest, by biomarkers, and by physical characters. Tumor-specific markers, for example, epithelial cell adhesion molecules (EpCAM), have widely been used for the detection of CTCs for epithelial tumors [11, 12]. This marker has been used in the Cell search system, a U.S. Food and Drug Administration (FDA)-approved clinical system to capture CTCs in blood in vitro, to capture EpCAM positive cells. However, the biomarkers may not be consistent in primary tumor cells and CTCs which undergo the epithelial-mesenchymal transition to down-regulate and even lose epithelial markers, present mesenchymal phenotype for better plasticity, migration, antiapoptosis, and invasiveness. Therefore, the

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technologies by biomarkers can hardly capture those CTCs with few markers from the original tumor. For nonepithelial tumors, there has not been a widely approved marker for most tumors yet and the heterogeneity of tumors and CTCs makes it more challenging to find specific biomarkers in common. The alternative to this biological idea is taking advantage of the size difference between CTCs and blood cells [13–15], given CTCs are usually larger than the major part of blood cells. By using microfluidic chips, CTCs, especially single CTCs, can be screened physically from blood. But the specificity of this technology is quite limited due to the variation in cell size of CTCs and blood cells. A significant limitation is those technologies only work in vitro, hindering deep insights into the kinetics and invasive mechanisms of CTCs in blood flow in vivo. Some instruments are developed by the idea of hemodialysis to enrich CTCs in vivo by using CTC filters (microfluidic chips), but the CTC detection cannot work in real time and the capture error is very large due to the size variation of cells.

By using the idea of in vivo flow cytometry [16, 17] that the blood vessels naturally work as fluidic systems and flowing cells inside can be excited by lasers noninvasively, the fluorescence of genetically-labeled CTCs in blood vessels can thus be detected in vivo in animal models. In this way, the kinetics of CTCs during the whole development of tumor can be monitored. Recently, we developed a scheme to use a confocal microscope system without any specific modification to detect CTCs flowing in blood vessels in vivo by performing continuous fast line scanning across the vessels. In this study, to investigate the relevance of CTC counts in blood and the final metastasis, we investigated the kinetics of CTCs in a tumor model of mouse at different heart rates by using the inhibitor of phosphodiesterase 3 and 4, isobutyl methylxanthine (IBMX) [18, 19] to tune the blood flow and increase the CTC counts without introducing significant side effects to the mouse. We found the metastasis was not influenced by the IBMX treatment although the detected CTCs in blood flow in this group significantly increased. Our results imply that the detected CTCs amount may not be directly related with metastasis.

2 | METHODS

2.1 | Cells

The prostate cancer cell line PC3 was generously provided by Prof. Weiliang Xia at Shanghai Jiao Tong University. PC3 cells were cultured in Roswell Park Memorial Institute-1640 medium containing 10% fetal bovine serum, 2 mM L-glutamine and 1% (vol/vol) penicillin/streptomycin at 37°C with 5% CO2. PC3 cells with stable expression of green fluorescent protein (GFP) and luciferase were cultured under the above culture conditions before tumor implantation.

2.2 | Animal models and tumor implantation

A total of 8 weeks male BALB/c nude mice weighing approximately 20 g were purchased from Charles River and maintained in a specific pathogen-free environment. For the tumor implantation, 100 μl cell suspension with the concentration of 10^7/ml PC3-GFP cells in phosphate-buffered saline was subcutaneously implanted into the axilla of right hind limb per mouse. During the initial 4 weeks of the tumor development, IBMX (1 μg/g, 430.622 μM, Sigma, I5879) was injected intraperitoneally twice a day into the mouse in the experimental group, the volume of tumor and the weight of mouse were measured twice a week. All animal experiments were performed in accordance with guidelines evaluated and approved by the Ethical Committee of Animal Experiments, Shanghai Jiao Tong University, China. The study was approved by the Ethic Committee of Shanghai Jiao Tong University (202101332).

2.3 | Heart rate measurement

A total of 8 weeks healthy male BALB/c nude mice were anesthetized with Isoflurane on the thermostatic stage and the heart rate was measured with the ultrasonic detector (JPD-200B1). Under the anesthetic, the rest heart rate was measured 3 times every 10 min first, and after the intraperitoneal injection of IBMX and PBS, the heart rate of mice was immediately measured every 10 min for the initial 2 h and every 30 min for the remaining 3 h. The mice were divided into four groups and injected with 0.2, 1, 5 μg/g IBMX and PBS respectively.

2.4 | CTC measurement

The 1 week after tumor implantation, CTCs of each mouse were measured with the confocal microscope system for 1 h once a week for 4 weeks. The mouse lay on its back on the microscope stage under the anesthetic and the ear was stuck on the glass slide with Polyethylene glycol to increase skin permeability. The blood vessels of mouse were stained with PE antimouse CD105 antibody (0.1 mg/ml, 100 μl, BioLegend, 120,408) by tail intravenous injection. The fluorescence of PE-CD105 and GFP-PC3 cells were respectively excited by 532 and 488 nm laser. Through 20X objective, an auricular vein near the base of ear was selected and a transverse scanning line was defined across it mapping to 256 pixels with a dwell time of 2 ms for continuous time-lapse loop. X and Y galvanoscopes coordinated with each other to construct a 2D image through time-interval line scanning on the path, and the x-t information of the passing CTCs was acquired. Finally, 3600 fluorescence images of 256 × 256 pixels were obtained by scanning the defined line in the defined time.

2.5 | In vivo bioluminescence imaging

On Day 31 after tumor implantation, in vivo bioluminescence imaging was performed. D-Luciferin potassium salt (10 μl/g, 15 mg/ml, Meilunstar, MB1834) a substrate of luciferase filtered by 0.2 μm filter membrane was injected to the caudal vein of mice 10 ~ 20 min before bioluminescence imaging to get the strongest fluorescence

signal. Mice were anesthetized with Isoflurane on the stage of animal in vivo imaging system (Parkin Elmer IVIS Lumina LT) to acquire signals of metastases from the whole body.

3 | RESULTS

3.1 | IBMX increases pacemaker activity of PC3 tumor mouse model

We at first investigated the effect of IBMX on the heart rate of mouse. Healthy mice (BALB/C, nude mice, male, 8 weeks) were intraperitoneally injected with IBMX (at $t = 20$ min) and their heart rate was continuously monitored for 5 h. It could be found in Figure 1A that after IBMX injection, the heart rate of those mice increased immediately, and peaked at ~70 min, which was greatly dependent on the dosage of IBMX (Control: $n = 4$ mice; IBMX 0.2 μg/g: $n = 3$; 1 μg/g: $n = 4$; 5 μg/g: n = 4 mice, respectively). The heart rate accelerated by IBMX at the concentration of 0.2 μg/g showed a significant increase compared with it of control but did not maintain for a long time. The mice injected with IBMX at 1 μg/g showed a relatively long and stable period of high heart rate (~ 380/min, >2 h). At 5 μg/g, the heart rate could increase to 400/min at the peak but the maintenance duration was exactly the same as it at 1 μg/g. As a compromise between the effective pacemaker acceleration and the side effect of this drug at high concentration, in this study, we used 1 μg/g IBMX to achieve a long-term and high increase in heart rate. After the implantation of the subcutaneous PC3 tumor (Day 0), the mice were injected with IBMX at 1 μg/g twice every day in the following 28 days, as in Figure 1B. The CTCs were detected in the auricular vein of mice for 1 h in vivo every week. On Day 31, the metastasis was examined by in vivo bioluminescence imaging.

GFP-luciferase-PC3 cells were implanted in the nude mice (8 weeks) at the axilla of each mouse's right hind limb (Control: $n = 6$, IBMX: $n = 11$ mice). The volume of the PC3 tumor was monitored during the tumor development. The tumor growth of the IBMX group was significantly slower than it of control from the second week (Figure 2A), probably because the enhanced metabolism induced by the high heart rate alleviated the hypoxic of tumor microenvironment and suppressed the release of inflammatory mediators and thus the tumor development. [20] Although the mice suffered tumor burdens, they still lived in good health and did not lose weight. The weight of mice from the two groups was very close to each other during the 4 weeks and consistently maintained the same as their original (Figure 2B). The IBMX-treated mice survived a little longer than the control, although no significant difference was found ($P = 0.066$), indicating the side effects of IBMX were quite few (Figure 2C).

3.2 | High CTC count is not related with metastasis

We then used a homemade inverted confocal microscope to monitor CTCs in the PC3 tumor mouse model in vivo [21]. The blood vessels were at first fluorescently labeled by tail intravenous injection of the vascular endothelial antibody, PE-CD105. The veins in the ear of mice were then localized by the fluorescence of vessel walls. We randomly selected a vein and defined a transverse scanning line across it to enable a continuous time-lapse confocal line-scanning loop, during which the fluorescence of genetically GFP-labeled CTCs in the blood flow could be excited by the scanning laser (488 nm) and detected (Figure 3A). As shown in Figure 3B, the morphology of blood vessels could be clearly acquired. The diameter of the vein was approximately 100 μm. The scanning line was then defined across it and mapped to 256 pixels with a dwell time of 2 ms per line. The total time of one-time line scanning was 3.04 ms (including the delay of galvomirror returning). Thus the continuous time-lapse laser scanning could achieve ~329 lines/s, which could capture all CTC passing through this scanning line with a speed of less than 15 mm/s. Since the blood flow speed in the small vessels of mouse was around 0.2 mm/s, all CTCs in such blood vessels could be captured [22, 23]. The typical CTC signal (GFP fluorescence) was shown as 2D images (the scanning line at the spatial dimension [x] and time-lapse scanning at the temporal dimension [t]) in the right panel in Figure 3B.

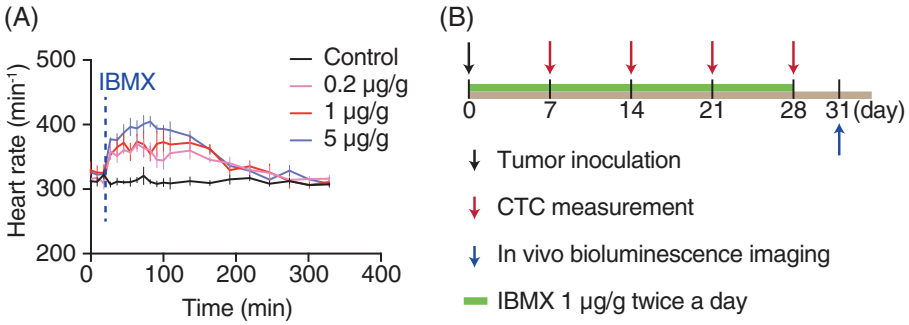

FIGURE 1 The heart rate of mice was increased by isobutyl methylxanthine (IBMX) for 28 days. (A) The heart rate versus time at different concentrations of IBMX. The IBMX was injected at 20 min (dashed line). (control: $N = 4$, 0.2 μg/g: $N = 3$, 1 μg/g: $N = 4$, 5 μg/g: $N = 4$) (B) The experimental scheme. The mice suffered IBMX twice a day for 28 days after tumor implantation (day 0). Using in vivo bioluminescence imaging of luciferase to examine the metastasis of tumor on day 31. CTC, circulating tumor cell. [Color figure can be viewed at wileyonlinelibrary.com]

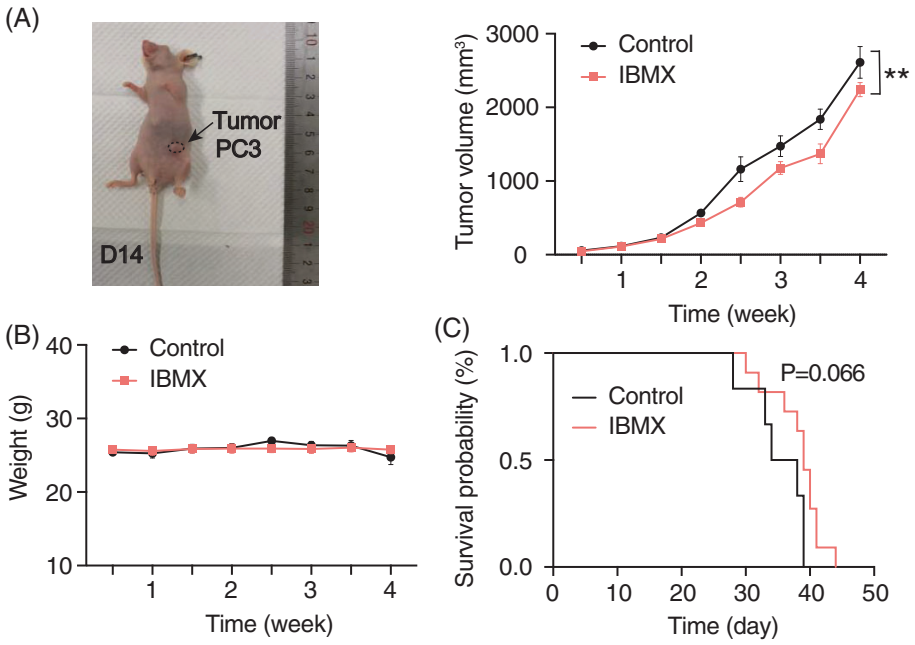

FIGURE 2 The PC3 tumor development. (A) The tumor volume of PC3 tumor mice with and without isobutyl methylxanthine (IBMX) injection along time (control: $N = 6$, IBMX: $N = 11$, P values were calculated by mix-effects analysis, $P = 0.006$). (B) The weight of mice along time (control: $N = 6$, IBMX: $N = 11$, P values were calculated by mix-effects analysis, $P = 0.943$). (C) The survival probability of mice (control: $N = 6$, IBMX: $N = 11$, P values were calculated by log rank, $P = 0.066$). Data represent mean ± *SEM*. $^{*}P < 0.05$. $^{**}P < 0.01$. [Color figure can be viewed at wileyonlinelibrary.com]

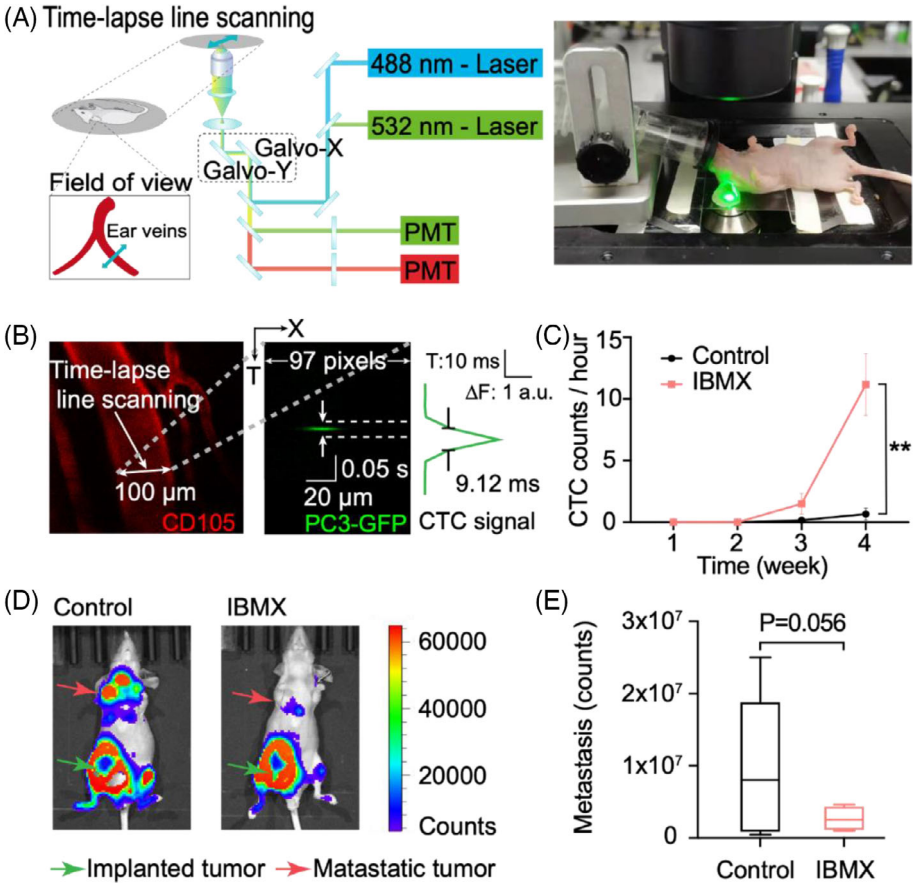

FIGURE 3 In vivo detection of circulating tumor cells (CTC) and metastasis. (A) The experimental setup to detect CTCs in vivo was based on a homemade inverted confocal microscope. (B) The fluorescent images of a blood vessel (left, red: CD105) and a CTC signal (green: green fluorescent protein [GFP]). The part of the scanning line across the vein (100 μm in the field of view) was mapped to 97 pixels for time-lapse scanning. (C) The CTC counts along time detected in the PC3 tumor mouse model with or without isobutyl methylxanthine (IBMX) injection (control: $N = 6$, IBMX: $N = 6$, P values were calculated by week4 unpaired t-test, $P = 0.0093$). (D) The in vivo bioluminescence imaging by luciferase for the GFP-luciferase-PC3 cells. (E) The quantified fluorescence intensity from (d) Excluding the preliminary tumors to indicate the metastasis (control: $N = 6$, IBMX: $N = 6$, P values were calculated by unpaired t-test, $P = 0.056$). Data represent mean ± *SEM*. $^{*}P < 0.05$. $^{**}P < 0.01$. [Color figure can be viewed at wileyonlinelibrary.com]

By this scheme above, we acquired the kinetics of CTCs during the development of PC3 tumor with a high heart rate induced by IBMX (as in Figure 1), which could theoretically increase the average CTC counts over time in blood. To verify this hypothesis, we examined the CTCs for 1 h every week along with the tumor development 12 h after the last IBMX injection trying to minimize the influence on the blood flow velocity caused by the increased heart rate (Control: $n = 6$, IBMX: $n = 6$ mice). It could be found that the CTC

counts in blood flow in those IBMX-treated mice were significantly higher than those of control since week 3 (Figure 3C). On Day 28, the detected CTC counts in the IBMX group were ~17 times of those in control. This result suggests that high heart rates could increase the CTC counts significantly.

We then examined the metastasis of mice of those two groups on Day 31 by using in vivo bioluminescence imaging of luciferase (Figure 3D). It could be found that the metastasis of the IBMX-treated mice that presented higher CTC counts in blood was even less than it of control ($P = 0.056$) as in Figure 3E. In this regard, the CTC counts detected in vivo were not directly related to the metastasis in this model.

4 | DISCUSSION

In this study, we used IBMX, a harmless drug to increase the pacemaker activity of mice to introduce higher CTC counts in blood during tumor development. We took advantage of fast line scanning of a confocal microscope to detect GFP-labeled CTCs in blood flow in vivo. The IBMX injection was found able to significantly increase the heart rate of mouse for more than 2 h. The CTC counts in those mice were significantly more than those of control. However, the metastases in those IBMX-treated mice were even less than those of control ($P = 0.056$). According to previous studies, IBMX treatments had no impact on the vasculature architecture and permeability, on the migratory and adhesive properties of tumor cells in vitro or on adhesion efficacies. [19].

There exist three hypotheses for this irrelevancy between CTC counts and metastasis. 1) IBMX only increased the blood flow to enable higher CTC counts over time. The total CTCs in IBMX and the control group were approximately the same. Under this condition, the CTC count was only a representation of the measurement but the metastasis, which might be determined mainly by the total CTCs, was thus not influenced. However, we measured the CTC counts 12 h after IBMX injection when the heart rate had dropped to the normal level. But the CTC counts were still significantly higher than those of control (~17 over 1). In this regard, this assumption could not be true. 2) The IBMX increased the blood flow and thus the shear force increased. The CTCs might be damaged by the high shear force of blood [24]. Hence even though the CTC count was high, the CTCs could not survive to migrate to organs and then form metastasis there. The CTC count was thus not related with metastasis. However, this possibility could not hold either considering the CTCs should not maintain a high level consistently for weeks if most of them were dead in blood. 3) The CTC count in blood was not related with metastasis. This could be plausible since metastasis might be mainly determined by CTC clusters that hold more stemness and survival ability [25–27]. Therefore, our results suggest a probability that all the detected CTC counts in blood, although it could work as an important biomarker of tumor development, might be irrelative to final metastasis. In general, our model and in vivo technology to detect CTCs also provide a non-invasive method for CTC and tumor research at in vivo level.

AUTHOR CONTRIBUTIONS

Hao He conceived and supervised the study. Xiaohui Zhao, Ziang Qi, and Ziao Gao performed the experiments. Xiaohui Zhao processed the data. All authors discussed, drafted, and revised the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

1. Chambers AF, Groom AC, MacDonald IC. Dissemination and growth of cancer cells in metastatic sites. Nat Rev Cancer. 2002;2(8):563–72.
2. Fabisiewicz A, Grzybowska E. CTC clusters in cancer progression and metastasis. Med Oncol. 2016;34(1):12.
3. Massagué J, Obenauf AC. Metastatic colonization by circulating tumour cells. Nature. 2016;529(7586):298–306.
4. Tayoun T, Faugeroux V, Oulhen M, Aberlenc A, Pawlikowska P, Farace F. CTC-derived models: a window into the seeding capacity of circulating tumor cells (CTCs). Cells. 2019;8(10):1145.
5. Krebs MG, Metcalf RL, Carter L, Brady G, Blackhall FH, Dive C. Molecular analysis of circulating tumour cells—biology and biomarkers. Nat Rev Clin Oncol. 2014;11(3):129–44.
6. Lianidou ES, Markou A, Strati A. The role of CTCs as tumor biomarkers. Adv Cancer Biomark. 2015;867:341–67.
7. Poudineh M, Sargent EH, Pantel K, Kelley SO. Profiling circulating tumour cells and other biomarkers of invasive cancers. Nat Biomed Eng. 2018;2(2):72–84.
8. Budd GT, Cristofanilli M, Ellis MJ, Stopeck A, Borden E, Miller MC, et al. Circulating tumor cells versus imaging—predicting overall survival in metastatic breast cancer. Clin Cancer Res. 2006;12(21):6403–9.
9. De Bono JS, Scher HI, Montgomery RB, Parker C, Miller MC, Tissing H, et al. Circulating tumor cells predict survival benefit from treatment in metastatic castration-resistant prostate cancer. Clin Cancer Res. 2008;14(19):6302–9.
10. Parkinson DR, Dracopoli N, Petty BG, Compton C, Cristofanilli M, Deisseroth A, et al. Considerations in the development of circulating tumor cell technology for clinical use. J Transl Med. 2012;10(1):138.
11. Deng G, Herrler M, Burgess D, Manna E, Krag D, Burke JF. Enrichment with anti-cytokeratin alone or combined with anti-EpCAM antibodies significantly increases the sensitivity for circulating tumor cell detection in metastatic breast cancer patients. Breast Cancer Res. 2008;10(4):R69.
12. Mamdouhi T, Twomey JD, McSweeney K, Zhang B. Fugitives on the run: circulating tumor cells (CTCs) in metastatic diseases. Cancer Metastasis Rev. 2019;38(1):297–305.
13. Xu L, Mao X, Imrali A, Syed F, Mutsvangwa K, Berney D, et al. Optimization and evaluation of a novel size based circulating tumor cell isolation system. PloS one. 2015;10(9):e0138032.
14. Xue P, Wu Y, Guo J, Kang Y. Highly efficient capture and harvest of circulating tumor cells on a microfluidic chip integrated with herringbone and micropost arrays. Biomed Microdevices. 2015;17(2):1–8.
15. Yu X, Wu N, Chen F, Wei J, Zhao Y. Engineering microfluidic chip for circulating tumor cells: from enrichment, release to single cell analysis. TrAC Trends Anal Chem. 2019;117:27–38.
16. Suo Y, Gu Z, Wei X. Advances of in vivo flow cytometry on cancer studies. Cytometry A. 2020;97(1):15–23.

17. Tuchin VV, Tárnok A, Zharov VP. In vivo flow cytometry: a horizon of opportunities. Cytometry A. 2011;79(10):737–45.
18. De Luca E, Zaccaria GM, Hadhoud M, Rizzo G, Ponzini R, Morbiducci U, et al. ZebraBeat: a flexible platform for the analysis of the cardiac rate in zebrafish embryos. Sci Rep. 2014;4(1):1–13.
19. Follain G, Osmani N, Azevedo AS, Allio G, Mercier L, Karreman MA, et al. Hemodynamic forces tune the arrest, adhesion, and extravasation of circulating tumor cells. Dev Cell. 2018;45(1):33–52.e12.
20. Boison D, Yegutkin GG. Adenosine metabolism: emerging concepts for cancer therapy. Cancer Cell. 2019;36(6):582–96.
21. Hu Y, Tang W, Cheng P, Zhou Q, Tian X, Wei X, et al. Monitoring circulating tumor cells in vivo by a confocal microscopy system. Cytometry A. 2019;95(6):657–63.
22. Yao J, Maslov KI, Shi Y, Taber LA, Wang LV. In vivo photoacoustic imaging of transverse blood flow by using Doppler broadening of bandwidth. Opt Lett. 2010;35(9):1419–21.
23. Zhou Y, Liang J, Maslov KI, Wang LV. Calibration-free in vivo transverse blood flowmetry based on cross correlation of slow time profiles from photoacoustic microscopy. Opt Lett. 2013;38(19):3882–5.
24. Leverett L, Hellums J, Alfrey C, Lynch E. Red blood cell damage by shear stress. Biophys J. 1972;12(3):257–73.
25. Au SH, Storey BD, Moore JC, Tang Q, Chen Y-L, Javaid S, et al. Clusters of circulating tumor cells traverse capillary-sized vessels. Proc Natl Acad Sci. 2016;113(18):4947–52.
26. Cheung KJ, Ewald AJ. A collective route to metastasis: seeding by tumor cell clusters. Science. 2016;352(6282):167–9.
27. Wang C, Mu Z, Chervoneva I, Austin L, Ye Z, Rossi G, et al. Longitudinally collected CTCs and CTC-clusters and clinical outcomes of metastatic breast cancer. Breast Cancer Res Treat. 2017;161(1):83–94.

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