Heterogeneity of circulating tumor cell dissemination and lung metastases in a subcutaneous Lewis lung carcinoma model

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Abstract: Subcutaneous (s.c.) tumor models are widely used in pre-clinical cancer metastasis research. Despite this, the dynamics and natural progression of circulating tumor cells (CTCs) and CTC clusters (CTCCs) in peripheral blood are poorly understood in these models. In this work, we used a new technique called ‘diffuse in vivo flow cytometry’ (DiFC) to study CTC and CTCC dissemination in an s.c. Lewis lung carcinoma (LLC) model in mice. Tumors were grown in the rear flank and we performed DiFC up to 31 days after inoculation. At the study endpoint, lungs were excised and bioluminescence imaging (BLI) was performed to determine the extent of lung metastases. We also used fluorescence macro-cryotome imaging to visualize infiltration and growth of the primary tumor. DiFC revealed significant heterogeneity in CTC and CTCC numbers amongst all mice studied, despite using clonally identical LLC cells and tumor placement. Maximum DiFC count rates corresponded to 0.1 to 14 CTCs per mL of peripheral blood. In general, CTC numbers did not necessarily increase monotonically over time and were poorly correlated with tumor volume. However, there was a good correlation between CTC and CTCC numbers in peripheral blood and lung metastases. We attribute the differences in CTC numbers primarily due to growth patterns of the primary tumor. This study is one of the few reports of CTC shedding dynamics in sub-cutaneous metastasis models and underscores the value of in vivo methods for continuous, non-invasive CTC monitoring.

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1. Introduction

Hematogenous metastasis is responsible for the majority of cancer-related deaths, wherein circulating tumor cells (CTCs) disseminate from the primary tumor via the peripheral blood (PB) [1]. CTC burden in PB is associated with metastatic progression, overall survival, and response to treatment for many cancers [2,3], both clinically and in pre-clinical small animal models [4–6]. CTCs are challenging to study because they are extremely rare. In fact, fewer than 1 CTC per mL of PB has been shown to have negative prognostic significance [7–9].

Biologists normally study CTCs using ‘liquid biopsy’ techniques, wherein blood samples are drawn, enriched, and CTCs isolated with assays such as flow cytometry, size-based cell separation, immuno-magnetic separation, or microfluidic capture [10]. For pre-clinical studies involving mice, the entire PB volume must often be drawn because CTCs are so rare. This inherently precludes longitudinal studies in the same mouse, making it extremely difficult (or impossible) to assess day-to-day changes in CTC numbers.

In vivo flow cytometry (IVFC) methods represent a class of bio-optical instruments that allow detection of circulating cells directly in the bloodstream, normally using either fluorescence or photoacoustic contrast [11,12]. These have the advantage of continuous, non-invasive monitoring of circulating cells over time [13]. Most fluorescence-IVFC methods use a modified confocal
microscope to detect moving cells in small blood vessels in the ear of a mouse where flow rates are about 1 µL PB per minute [14,15]. Therefore, microscopy-IVFC methods are not readily applicable to CTC research unless the CTC burden is very high, e.g. in particularly invasive metastasis models [16]. For example, the presence of 1 CTC per mL of PB would yield only about one detection in 1000 minutes of data acquisition.

To address this issue, our team recently developed a new fluorescence-IVFC technique called ‘diffuse in vivo flow cytometry’ (DiFC) [17–22]. DiFC uses diffuse photons to sample up to hundreds of µL per minute of circulating PB in large, deep-seated blood vessels. Previously, we showed that DiFC allows detection of early-stage dissemination of green-fluorescent protein (GFP)-expressing CTCs in a multiple myeloma (MM) xenograft model at burdens as low as 1 CTC per mL of PB [17].

In the present study we used DiFC to investigate the dynamics of CTC dissemination in a widely used subcutaneous (s.c.) Lewis lung carcinoma model [23–32]. The novelty of this work is two-fold. First, to our knowledge there have been no previous reports of CTC dynamics in s.c. LLC tumors. In fact, despite their broad usage in small animal cancer and metastasis research [25–29,33–37], there have been few reports of changes in CTC numbers during tumor growth (as opposed to terminal blood draws at study endpoints) for any s.c. models [23,28,38]. Even fewer studies have studied CTC clusters (CTCCs) [39], which are of increasing interest since they are known to have significantly higher (100 times) likelihood of forming metastases than individual CTCs [6,38,40,41]. To re-iterate, the major technical challenge is the low abundance of CTCs in these models. For example, Fan et. al. used fluorescence-microscopy IVFC to detect CTCs in an s.c. hepatocellular carcinoma model, and observed count rates of only few cells per hour when tumor volumes reached 6000 mm³ [42].

Second, s.c. LLC tumors are known to spontaneously metastasize to the lungs and in some cases the liver via the vasculature [25,28,43–47], often with significant inter-mouse variability. In previous work, this heterogeneity has usually been attributed to the appearance of clonal sup-populations or cell phenotype, such as the low metastatic phenotype (LLC-LM) [24,28,32,47]. Despite the use of clonally identical cells and nominally identical tumor placement, we also recorded significant inter-mouse variability in CTC and CTCC numbers. These generally correlated to different rates of lung metastasis formation measured using bioluminescence imaging (BLI) at the study endpoint. Our data suggests that the heterogeneity in metastatic spread likely originated from small differences in tumor placement and access to vasculature.

2. Materials and methods

2.1. DiFC instrument and data analysis

We used our GFP-compatible DiFC instrument (Fig. 1(a)) that was described in detail previously [17,20]. Briefly, DiFC uses two custom-designed fiber optic probes that are placed on the skin surface. For the experiments here, the probes were placed on the ventral side of the mouse tail, approximately above the ventral caudal bundle (Fig. 1(b)). Tissue was illuminated with 488 nm laser light, allowing transient fluorescence detection of GFP-expressing LLC cells as they passed through the DiFC field of view (Fig. 1(c,d)). DiFC data was acquired at a sampling rate of 2000 samples/s.

DiFC data was analyzed using the same approach described in Patil et. al. previously [20]. Raw data from the two fibers (channels 1 and 2) was first pre-processed by background subtraction followed by the application of a 5 ms moving average filter. Peaks exceeding 6 times the background standard deviation (calculated on a moving basis for each minute of data) were identified as peak candidates. Coincidental peak candidates (detected simultaneously on both channels) were discarded on the assumption that they were noise due to motion or electronic artifacts. We sometimes observed differences in count rates between the two fiber bundles. This difference was presumably due to small differences in surface alignment and probe positioning.
over the blood vessel, which may have impacted fluorescence light collection. After analyzing both channels independently, we took the higher of the two count rates on a given day, on the assumption that this was the better aligned fiber. As we show, these differences were generally small.

As discussed in more detail in the results section below, we used the amplitude of the peaks (measured in mV) to estimate the number of cells in each detection. As in our previous work [17], we sometimes detected large peaks which we attributed to multicellular groupings of CTCs (CTCCs). In signal processing, we defined a CTCC as a peak with amplitude at least 3 times that of a single cell. As we show, the latter was empirically determined to be approximately 15 mV, so that a CTCC-like signal was defined here as a peak exceeding 45 mV.

2.2. Lewis lung carcinoma cells

We used LLC cells that were genetically modified to express luciferase and green fluorescent proteins (LL/2-Fluc-Neo/eGFP-Puro, Imanis Life Sciences, Rochester, MN). Cells were grown at 37°C in 5% CO₂ in DMEM with 10% fetal bovine serum plus 1% penicillin-streptomycin, 2 µg/mL puromycin, and 1.25 mg/mL G418 (Sigma Aldrich, St. Louis, MO) under sterile culture conditions. Addition of G418 antibiotic continuously selected for GFP-expressing LLC cells, and no additional sorting of cells for brightness was performed. It is a property of stably-transfected GFP-expressing cell lines that all daughter cells retain GFP expression, making them extremely valuable in studying growth and metastatic spread in pre-clinical mouse models as in the study here.
We first tested GFP expression of the cells by flow cytometry (Attune NXT, ThermoFisher Scientific, Waltham, MA). We compared the fluorescence brightness to Dragon Green (DG) reference fluorescent microspheres (DG06M, Bangs Laboratories, Fishers, IN). DG beads are sold in sets of five calibrated intensities, increasing from DG1-DG5. Our previous data showed that DiFC is capable of detecting CTCs in nude mice at brightness levels exceeding DG3 [17]. As shown in Fig. 2, a distribution of GFP expression was observed, reflecting cells at different points in the cell division cycle. The majority (∼70%) of LLC cells expressed GFP exceeding the brightness of DG3 beads, indicating that they should be readily detectable in mice in vivo (whereas the lower GFP-expressing cells are likely undetectable).

![Fluorescence histogram of LL/2-Fluc-Neo/eGFP-Puro (LLC) cells (red) and DG3 reference microspheres (blue). The majority (70%) LLC cells expressed GFP at brightness exceeding DG3, indicating that they should be readily detectable with DiFC in circulation in mice in vivo.](image)

2.3. Subcutaneous LLC tumor model

8-week-old female Athymic NCr-nu/nu mice (Charles River Labs, Cambridge, MA) were used for this study. Animal handling and experimental procedures conformed to NIH guidelines and Northeastern University’s Institutional Animal Care and Use Committee (IACUC) policies (protocol# 15-0728R). Mice were caged in groups of five or less, and all animals were fed a diet of low fluorescence animal chow (AIN 93M Mature Rodent Diet, Ziegler Feed, East Berlin, PA)

Mice were maintained under inhaled isofluorane (to effect) during DiFC scanning to prevent movement, and were kept warm with two heating pads, one under the body and the other over the exposed area of the tail.

As an initial test to verify that the GFP+ LLC cells could be detected by DiFC in vivo, we performed direct intravenous injection of 5×10^5 cells suspended in 100 µL of PBS via the tail vein. Cells were harvested from culture with a cell scraper and were not trypsonized, to avoid modification of cell membrane proteins or alteration of the kinetics of clearance from circulation. We performed DiFC scanning on the mouse tail, beginning 10 minutes after injection for a total of 2 hrs.

Next, s.c. LLC tumors were established in nude mice (N = 11) as previously reported [47]. 10^6 cells were suspended in 100 µL DMEM and injected s.c. in the right hind limb of the mice.
Mice were scanned with DiFC regularly for 60 minutes approximately once per week during tumor development. Tumor size (length L, width W) were measured with calipers to estimate the volume as \( V = L \cdot W \cdot W/2 \). Mice were euthanized when one of the following criteria were met: i) tumor exceeded volume of 3000 mm\(^3\), ii) ulceration of surrounding skin above or surrounding the tumor was observed, or, iii) mice appeared moribund (>15% loss of body weight, hunched posture, labored breathing, body condition score < 2).

We also performed regular DiFC on un-injected control mice (N = 6) throughout the study. Control mice were scanned for 30 minutes on the same days as the tumor-bearing mice for a combined total of 17 hours of scanning. The rationale was that any DiFC detections in these mice were false positive signals, which we used to determine the false-alarm rate (FAR) of the system throughout the study period.

2.4. Excision of lungs and bioluminescence imaging

Immediately following euthanasia, we harvested the lungs from 9 of the LLC tumor-bearing mice and 6 control mice. The excised lungs were soaked in a 3 mg/mL solution of D-Luciferin (Xenolight D-Luciferin, Perkin Elmer, Boston, MA) in PBS for 5 minutes. Bioluminescence imaging (BLI) was performed on the lungs using an IVIS Lumina II imaging system (Caliper Life Sciences, now Perkin Elmer, Waltham, MA). For one tumor-bearing mouse the lungs were accidentally damaged during excision and could not be imaged. The BLI imaging exposure was 1 second. Images were analyzed using mean flux (photons/second) in regions of interest (ROIs) drawn around each set of lungs.

2.5. Cryomacrotome fluorescence imaging

We performed cryo-macrotome imaging in a limited study of two LLC-bearing mice which (as we discuss) displayed drastically different rates of CTC shedding. This allowed us to visualize the fluorescence distribution of GFP-expressing cells in the whole body. Each mouse was euthanized, its bottom teeth removed, and then the mouse was immediately placed in a 50 mL conical tube. The entire tube was filled with optimal cutting temperature (OCT) compound (Fisher Healthcare, Inc., Waltham, MA) and then flash frozen in liquid nitrogen. Samples were stored at −80°C prior to imaging.

A hyperspectral imaging whole body cryo-macrotome instrument was described previously [48]. Briefly, the system operates by automatically sectioning frozen specimens in a slice-and-image sequence, acquiring images of the specimen block after each section is removed. The acquired image stacks were combined in open source software platform (NIRFAST-Slicer 2.0) to generate high-resolution three-dimensional volumes of the brightfield and fluorophore distribution throughout whole body animal models. For this study, we acquired brightfield and GFP fluorescence volumes of each animal at a resolution of 150 µm in the sectioning direction and ~100 µm in the imaging plane.

3. Results

3.1. Detection of LLC cells in circulation with DiFC

We first verified that GFP+ LLC cells were detectable with DiFC in mice by direct tail vein injection as shown in Fig. 3. A representative 10-minute DiFC data trace obtained from a control (un-injected and non-tumor bearing) mouse is shown in Fig. 3(a). As in our previous work [17,18], the DiFC signal was generally very stable after background subtraction, leading to very few false positive counts. Specifically, in 17 hours of DiFC scanning on control mice performed throughout the course of this study, the standard deviation of the background-subtracted signal was on average \( \sigma = 1.1 \) mV. Using the signal processing approach described in section 2.1 above, the false-alarm rate (FAR) was on average 1.4 per hour.
A representative 10-minute DiFC data trace from a mouse injected i.v. with $5 \times 10^5$ LLC cells via the tail vein is shown in Fig. 3(b). Each ‘peak’ in the plot represents an LLC cell (or multi-cellular grouping of cells) passing through the DiFC field-of-view. These were clearly observable above the DiFC background.

The distribution of peak amplitudes measured over a single 2-hour scan is shown in Fig. 3(c). As in our previous work [17,18], this distribution stems from two sources, i) small variations in depth of the CTC traveling in the blood vessel, and ii) variability of GFP-expression of CTCs. For the former, the ventral caudal artery is about 250 $\mu$m in diameter and about 1 mm deep in the tail. Our previous Monte Carlo analysis of blue and green light propagation in DiFC [17] suggests that this effect would cause a measured intensity difference by at most a factor 2.5 between the upper and lower walls of the vessel. For the latter, the flow cytometry analysis shown in Fig. 2 suggest that GFP expression differences may account for as much as an order of magnitude difference in detected intensity, and is therefore likely the more significant of the two effects.

The most common (mode) peak amplitude was 15 mV. We subsequently took this to be the mean amplitude of a single LLC cell passing through the DiFC field of view. Given the measured background standard deviation in un-injected mice (average of $\sigma = 1.1$ mV), the signal-to-noise ratio (defined as $\text{SNR} = 20 \log_{10}(I/\sigma)$) for a single LLC cell was approximately 23 dB.

Larger peaks were also observed, in this case with amplitudes up to 1000 mV (59 dB). Based on our previous work in MM mice [17], we identified these as multi-cellular LLC aggregates (CTCCs). The presence of multicellular aggregates is unsurprising since LLC cells are highly...
adherent and readily form groupings in culture. As noted, we did not trypsinize the cells prior to the injection, meaning that adherent cell surface molecules were intact.

Figure 3(d) shows a raster (event) plot of DiFC detections as a function of time after injection of LLC cells. Cells were detected for at least two hours after injection, after which we stopped acquiring data. The average DiFC count rate steadily declined over the scan (the 10-minute moving average count rate is shown Fig. 3(e)). The final count rate after 2 hours was approximately 25% of the initial value, meaning that the CTC half-life in circulation was approximately 1 hour. Although it is highly likely that many of the initially injected LLC cells cleared rapidly from circulation on the first pass through the lungs [49,50], this confirms that at least some of the injected cells remained in circulation for an extended period of time. We note that there were only minor differences in the median and mean peak amplitudes between the first and second hours of the DiFC scan (first hour: mean = 60.3 mV, median = 20.5 mV; second hour: mean = 78.1 mV, median = 17.4 mV). This implies that there was negligible photobleaching of the GFP-expressing CTCs, which is supported by the short (~10s of ms) dwell time of CTCs as they pass through the DiFC field of view [17,18].

3.2. LLC tumors and growth

Subcutaneous LLC tumors were next established in nude mice. Representative tumor images are shown in Fig. 4(a). Tumors grew rapidly in all mice (Fig. 4(b)), reaching the size limit of 3000 mm$^3$ in 10 of 11 mice in 29.2 ± 2.7 days (we note that this tumor size limit was allowed under our protocol since CTCs and lung metastases were typically not detectable in this model until tumors grew in excess of 1000 mm$^3$). One mouse (of 11) had skin ulcerations and was euthanized early on day 25. None of the mice appeared moribund.

![Fig. 4. Growth of s.c. LLC tumors in mice. (a) Representative photographs of tumor growth. (b) The tumor volume following inoculation for all N=11 mice in this study. Red curves indicate HS (3 of 11) mice where relatively high CTC numbers were measured with DiFC. Blue curves indicate LS (8 of 11) mice for which CTC numbers were relatively low.](image)

3.3. Measurement of CTCs with DiFC during LLC growth

We performed 60-minute DiFC scans on LLC tumor-bearing mice. One of the striking features of the data was the inter-mouse variability. Based on literature data [6–8,40,51–57] and our previous work with an MM xenograft model [17], we anticipated a steady increase in CTC numbers as tumors grew. In fact, only 3 of 11 mice studied exhibited this general pattern (labeled in red on Fig. 4(b)). We term this subset of mice “high shedding” (HS) which we defined as,
i) exhibiting a monotonic increase in CTC numbers with time (tumor volume), and, ii) having final DiFC count rate exceeding 20 detections per hour (approximately 6.7 CTCs per mL PB). Representative 60-minute DiFC data traces measured on different days from an HS mouse are shown in Figs. 5(a)-(d).

![DiFC data traces](image)

**Fig. 5.** (a-d) Representative DiFC data from an example HS mouse where CTC numbers increased steadily over time. The tumor volume (TV) on each day is also indicated. (e-h) Representative DiFC data from an example LS mouse where CTC numbers remained low throughout the study. Red circles indicate cell detections.

However, for the majority of the mice (8 of 11) CTC numbers did not exhibit a monotonic increase with time (tumor volume), but rather were often observed on one day, and not at all in a subsequent scan. In addition, CTC count rate (numbers) were poorly correlated with tumor volume and remained relatively low throughout the study. We termed these mice “low-shedding” (LS). LS mice are labeled in blue in Fig. 4(b), and as shown there were no obvious differences in rates of tumor growth. Representative 60-minute sequences of DiFC data collected from a LS mouse are shown in Figs. 5(e)-(h).

We also measured large fluorescent peaks in 5 of 11 mice which we (again) attributed to CTCCs. In general, these were observed when tumors were large. Specifically, >90% were detected when volumes exceeded 2000 mm$^3$, and 100% were detected when tumor volumes exceeded 1000 mm$^3$. This is in agreement with Suo et. al. [53], who observed that the number of CTCC detections increased with increasing tumor volume. Overall, CTCCs were detected at a frequency of 7.9% of all CTC detections.

With respect to cluster sizes, the histogram of peak amplitudes is shown in Fig. 6(a). As with the direct i.v. injection experiment (Fig. 3(c)), the mode peak amplitude was 15 mV, which is our best estimate of the amplitude of a single LLC cell. From this, we estimated the number of cells for each detected peak (red axis Fig. 6(a)). No peaks exceeded 150 mV in amplitude, which was equivalent to approximately 10 aggregated LLC cells, and most groupings skewed towards smaller cluster sizes (3-4 cells). This is in good general agreement with the size distribution of clusters in human melanoma, prostate, and breast cancers summarized in [58].

Based on these data, we computed the “corrected” equivalent DiFC count rate per hour by summing the contributions from single cells and CTCCs. As noted above, CTC count rates in
Fig. 6. (a) Histogram of the peak amplitudes for all CTCs detected throughout the study. (b) Corrected CTC detection rates per hour (see text for details) compared to the tumor volume. (c-m) Individual plots of corrected CTC detection rates per hour vs. days post inoculation. Blue plots indicate LS mice and the red plots represent HS mice. The higher count rate measured on the two fiber bundles (closed symbols) was used in subsequent calculations. In some cases this was higher than the average of the two (open symbols), presumably due to small differences in surface alignment and geometrical collection of fluorescence. The black dotted lines show the DiFC false alarm rate measured during this study in control mice.

HS mice generally correlated with tumor volume ($r^2 = .616$; red points, Fig. 6(b)), while little correlation between tumor volume and CTC count rate was observed for the LS mice ($r^2 = .211$; blue points). The corrected DiFC count rates as a function of time for LS and HS mice are shown in Figs. 6(c)-(j) and Figs. 6(k)-(m), respectively. As noted, there was sometimes a small difference between the count rate measured between the two fiber bundles which we attribute to small differences in probe alignment on the skin surface. In calculation of the DiFC count rate, we took the maximum of the two signals (closed symbols) as opposed to the average (open symbols shown for comparison), on the assumption that the probe was better aligned. As shown, these differences did not affect general trends in the data. Although not used in this study, we subsequently redesigned the DiFC probe holder so that each fiber probe could be independently adjusted during alignment. This further mitigated these differences.
3.4. BLI imaging of LLC tumor in lungs

At the study endpoint, mice were euthanized, and 8 of the lungs were excised and imaged with BLI (one set of lungs from an LS mouse was accidentally damaged during excision and could not be imaged). Representative lung images from control and LLC tumor-bearing mice are shown in Figs. 7(a) and (b) and Figs. 7(c) and (d), respectively. By visual inspection, discoloration and red nodes were observed on some of the LLC tumor-bearing lungs, but generally the lungs were free of large visible tumor masses. BLI revealed (again) heterogeneous lung metastasis formation. A relatively small number of large metastatic nodes were observed by BLI in 5 out of 8 lungs, although all lungs from tumor-bearing mice displayed at least some BLI signal above the background measured in control mice. Figure 7(e) shows the median BLI signal for control and tumor bearing mice. We also compared the final DiFC-measured CTC count rate against the BLI signal for each mouse as shown in Fig. 7(f). With the exception of one mouse, LS mice generally exhibited much lower BLI signal than HS mice.

Because CTCCs are known to have higher metastatic potential than single CTCs, we considered whether these also contributed to higher lung BLI signal. Mice where CTCCs were detected are indicated with a (+) symbol in Fig. 7(f), whereas mice were no CTCCs were detected are indicated with circles. As shown, the presence of CTCCs generally corresponded to higher BLI signal. Overall, these data suggest association between CTC and CTCCs measured with DiFC and lung metastases measured with BLI.

Last, noting the large range of CTC and CTCC shedding in the mice for nominally identical experiment repeats, we performed whole body cryo-macrotome imaging of the tumor region.

Fig. 7. Example (a) photograph and (b) BLI image of excised lungs for a control mouse. (c) Photograph and (d) BLI image of lungs from an LLC-tumor bearing mouse. Lung metastases appeared as a red discolored region on white light images of lungs (red circle, panel (c)) and produced BLI signal. (e) Average BLI signals (counts s^{-1}) obtained from control (N=6) and tumor-bearing (N=8) mice. Although the median BLI signal for the LLC group exceeded that of the controls, some tumor bearing mice showed little increase over background. (f) The average BLI signal as a function of the DiFC count rate measured on the final day (prior to euthanization) for each mouse is shown. Blue symbols indicate LS mice, whereas red symbols indicate HS mice, showing a general agreement between CTC numbers and the presence of lung metastases. (+) symbols denote mice for which CTCCs were detected and BLI imaging was performed.
of an LS and an HS mouse. Figures 8(a) and (b) show example fluorescence images (coronal and 3-dimensional reconstruction overlaid on white-light images) for the LS mouse, which showed little or no infiltration into the peritoneal cavity. Conversely significant invasion into the peritoneal cavity was observed for the HS mouse as shown in Figs. 8(c) and (d). Since the peritoneal cavity is well-vascularized, we posit that this growth pattern largely accounted for differences in the CTC detection rates between the two mice. As discussed in more detail below, these data suggest that the large differences in CTC detection rates and lung metastasis formation in the HS and LS groups may be due to differences in tumor invasion patterns.

4. Discussion and conclusions

In this work, we used DiFC to monitor CTC and CTCC dissemination in an s.c. LLC tumor model in mice for the first time. DiFC allowed us to monitor day-to-day changes in CTCs during tumor development. To reiterate, the principle advantage of DiFC compared to microscopy-based IVFC methods is that it samples at least an order of magnitude higher circulating PB volume and
therefore allows detection of very rare CTCs [17]. This is particularly important in the present s.c. LLC model, where the measured DiFC count rate usually did not exceed 20 CTCs/hour, or approximately 6.7 cells/mL of PB. The rarity of LLC cells means that CTCs would have been detectable only at late stages of growth by analysis by liquid biopsy of PB samples. These would not allow profiling of the natural course of CTCs over tumor development in individual mice as we have done here. Methods involving blood collection are also generally blind to the intermittent nature of CTC and CTCC shedding as in this model, i.e. since blood is only drawn at a small number of time points.

The most notable feature of these data is that nominally identical experimental repeats yielded large heterogeneity in CTC numbers detected with DiFC. In general, CTC numbers did not necessarily correlate with tumor volume, but were generally associated with the formation of metastases (measured by BLI). Due to the intermittent nature of CTC shedding this model, it is probable that shedding bursts were simply missed based on when DiFC was performed, i.e. at different times on different days.

Whole body cryo-macrotome images of two of the mice suggest that differences in CTC rate and metastasis formation may simply be due to different growth patterns of the primary tumor, as opposed to genetic sub-populations as others have proposed [23,27,31,47]. These may have originated from small differences in for example, initial tumor placement, access to vasculature, surrounding microenvironment and tissue. This is an area of more detailed study in our group in the future, and more detailed genetic characterization of primary tumors and metastases would be needed to confirm this hypothesis.

DiFC also revealed the presence of LLC CTCCs in PB for a relatively small subset of mice (5 of 11). CTCCs appeared with a frequency of 7.9% of all CTC detections, and almost always when tumor volumes were large (>2000 mm³). This is consistent with previous mouse models where an increase in the fraction of CTCCs were reported for larger tumor volumes [53]. Again, due to their rarity the use of liquid biopsy of small blood samples would likely have ‘missed’ detection of CTCCs entirely. To further investigate this, we plan to improve our DiFC instrumentation and setup to allow continuous monitoring of CTCs and CTCCs in mice over longer time periods future studies.

In conclusion, we demonstrated a novel application of DiFC for a widely-used mouse tumor model, where the dynamics of CTC and CTCC numbers in peripheral blood have not been previously reported. These data underscore the value of high-sensitivity in vivo methods for CTC detection such as DiFC. Ongoing work in our group involves application of DiFC to other tumor models, and study of the CTC numbers in response to anti-cancer therapies [47].

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