Published in final edited form as:

Mol Imaging Biol. 2020 October ; 22(5): 1280–1289. doi:10.1007/s11307-020-01505-9.

Fluorescence Labeling of Circulating Tumor Cells with a Folate Receptor-Targeted Molecular Probe for Diffuse *In Vivo* Flow Cytometry

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Abstract

Purpose: We recently developed a new instrument called “diffuse *in vivo* flow cytometry” (DiFC) for enumeration of rare fluorescently labeled circulating tumor cells (CTCs) in small animals without drawing blood samples. Until now, we have used cell lines that express fluorescent proteins or were pre-labeled with a fluorescent dye *ex vivo*. In this work, we investigated the use of a folate receptor (FR)-targeted fluorescence molecular probe for *in vivo* labeling of FR+ CTCs for DiFC.

Procedures: We used EC-17, a FITC-folic acid conjugate that has been used in clinical trials for fluorescence-guided surgery. We studied the affinity of EC-17 for FR+ L1210A and KB cancer cells. We also tested FR– MM.1S cells. We tested the labeling specificity in cells in culture *in vitro* and in whole blood. We also studied the detectability of labeled cells in mice *in vivo* with DiFC.

Results: EC-17 showed a high affinity for FR+ L1210A and KB cells *in vitro*. In whole blood, 85.4 % of L1210A and 80.9 % of KB cells were labeled above non-specific background with EC-17, and negligible binding to FR– MM.1S cells was observed. In addition, EC-17-labeled CTCs were readily detectable in circulation in mice with DiFC.

Conclusions: This work demonstrates the feasibility of labeling CTCs with a cell-surface receptor-targeted probe for DiFC, greatly expanding the potential utility of the method for pre-clinical animal models. Because DiFC uses diffuse light, this method could be also used to enumerate CTCs in larger animal models and potentially even in humans.

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Conflict of Interest

Philip S. Low is on the Board of Directors for On Target Laboratories Inc., manufacturer of EC17 and OTL38.

Compliance with Ethical Standards All mice were handled in accordance with Northeastern University’s Institutional Animal Care and Use Committee (IACUC) policies on animal care. Animal experiments were carried out under Northeastern University IACUC protocol #15-0728R. All experiments and methods were performed with approval from and in accordance with relevant guidelines and regulations of Northeastern University IACUC.

Keywords

Circulating tumors cells (CTCs); Folate receptor; Diffuse imaging; Fluorescence; *In vivo* flow cytometry

Introduction

Cancer metastasis is a multi-step process, by which tumor cells colonize distant organs and tissues. The circulatory system is one of the most common pathways, wherein tumor cells intravasate into the peripheral blood, circulate, and form metastases at secondary sites [1–3]. Circulating tumor cells (CTCs) in the bloodstream are therefore of great interest in cancer research. Numerous studies have demonstrated that CTC numbers correlate with overall survival, disease progression, and response to treatment for many cancers [4–9]. CTCs are extremely rare: fewer than 1 CTC per milliliter of peripheral blood is considered prognostically negative [10].

Normally, CTCs are studied using “liquid biopsy,” where relatively small blood samples are drawn from the patient or small animal (in the case of pre-clinical research), are purified, enriched, and further analyzed [11, 12]. Although these are widely used in biomedical research, they are far from optimal for a number of reasons. In particular, small blood samples provide a poor statistical sampling of the circulating blood volume [13–15], blood is known to degrade rapidly after removal from the body [16], and enrichment can cause cell loss or dissolution [17, 18]. Moreover, in the case of small animal studies, CTCs may be so rare that it is necessary to draw and analyze the entire blood volume, which requires euthanizing the animal [19] and therefore precludes the longitudinal study of the same animal over time.

These limitations have driven the development of optical methods for enumerating circulating cells without having to draw blood samples, collectively termed “*in vivo* flow cytometry” (IVFC) [20–23]. IVFC typically uses specialized confocal microscopy [24–27] or photoacoustic [28–30] instrumentation to detect circulating cells, for example, in a small blood vessel in the ear of a mouse. Our group recently developed “diffuse *in vivo* flow cytometry” (DiFC) [31–34], a new technique for counting rare fluorescently labeled CTCs with highly scattered light. This allows sampling of large circulating blood volumes (compared with a microscope) and therefore detection of less-abundant cells. For example, we recently used DiFC to monitor the dissemination of multiple myeloma cells in a xenograft model [33] and showed that we could non-invasively detect fewer than 1 CTC per milliliter of blood. In addition to detection sensitivity, a major advantage of DiFC is that it works in bulk, optically diffusive tissue such as the mouse leg or tail (as opposed to the thin ear of a mouse as in microscopy) so in principle could be used in larger limbs and species. However, until now, we have only used DiFC with CTCs that express green fluorescent proteins (GFP) or by labeling cells *ex vivo* with membrane or cytosol dyes. This presently limits the use of DiFC to the study of mouse xenograft models using cultured immortalized cell lines [32, 33] or transgenic mouse models that express fluorescent proteins.

Use of a targeted fluorescent molecular probe that could label CTCs while in circulation could therefore greatly expand the utility of DiFC. In this work, we studied a folate receptor (FR) alpha (α)-targeted molecular probe for DiFC for the first time. FR is widely used as a therapeutic and diagnostic target for cancer since it is often overexpressed in many epithelial cancers [35–38], including ovarian [39–42], breast [43, 44], and non-small cell lung carcinomas [45, 46]. FR is purported to have very low expression in normal tissues [35–37]. Previous work has shown that FR overexpressing cancer cells may express 10^6 FR per cell, whereas most normal cells express fewer than 5×10^4 FR per cell, and activated monocytes 10^5 FR per cell [47, 48]. This suggests that FR is a promising molecular target for DiFC labeling.

Cell-surface receptor-targeted molecular contrast agents have already been used for circulating cells in microscopy IVFC previously by others [22, 49] including for FR+ cells [24, 47]. However, a major question here was whether CTCs could be labeled with sufficient specificity and brightness for detection with DiFC. DiFC uses diffuse light and is therefore more susceptible to scatter and attenuation of biological tissue (compared with microscopy) [50] and as such has higher fluorescence labeling requirements [22, 32].

In this work, we tested the EC-17 FR α -fluorescent probe. EC-17 is a small-molecule FITC-based probe that was originally developed for fluorescence-guided surgery (FGS) and has been shown to have a high affinity for FR+ CTCs previously [47]. Moreover, EC-17 and its NIR analog OTL-38 have both been used in FGS clinical trials [39, 51, 52], leading to the exciting possibility of using DiFC in humans in the future. We tested EC-17 with two FR+ human cancer cell lines. As we show, EC-17 had very sensitivity and specificity for FR+ CTCs, and labeled CTCs were detectable in mice *in vivo* with DiFC. Overall, this work demonstrates the feasibility of using targeted molecular probes for DiFC for the first time, which could greatly extend its utility in CTC research.

Materials and Methods

Diffuse In Vivo Flow Cytometry

The DiFC instrument is shown in Figs. 1a, b. The design and signal processing algorithms were described in detail by us previously [32, 33]. Briefly, DiFC works on the principle of laser-induced fluorescence. The DiFC fiber probes (red arrows, Fig. 1c, d) have integrated optical filters and lenses that allow detection of the weak fluorescence signal from individual moving cells in blood [34]. When placed over a major blood vessel, for example, in the tail or leg (Fig. 1c) of a mouse, transient fluorescence peaks are detected and counted. We showed previously that the ventral caudal artery in a mouse tail carried hundreds of microliters of blood per minute [32], allowing us to detect and count very rare circulating cells [33].

We previously built blue (b-DiFC) and red (r-DiFC) versions of DiFC [32, 33]. b-DiFC is compatible with absorption and emission spectra of EC-17 (see Folate Receptor-Targeted Fluorescent Molecular Probes). A number of minor hardware improvements were also made to the b-DiFC system here. First, we used voltage output photomultiplier tubes (Hamamatsu H10722–20; Edmund Optics, Barrington, NJ) and voltage pre-amplifiers (SR650; Stanford

Research Systems, Sunnyvale, CA) rather than current output PMTs, due to improved noise properties. Second, we added a second, 536-nm bandpass interference filter (IDEX Health and Science, LLC, Rochester, NY) in front of each PMT to improve out-of-band blocking.

Folate Receptor-Targeted Fluorescent Molecular Probes

EC-17 is a small-molecule FR α -targeted probe (MW 917 Da) which was developed in the lab of Prof. Low and has been extensively characterized previously [47, 53, 54]. EC-17 is a conjugation of fluorescein isocyanate (FITC) to folic acid, which is the binding ligand of the folate receptor. Previous work showed that EC-17 has an excellent affinity for FR+ CTCs in blood compared with larger antibody-based probes [24, 47]. The EC-17 maximum excitation and emission wavelengths are 490 nm and 520 nm, respectively.

Cell Lines

KB is an FR+ HeLa-derived human cervical cancer line that we purchased from ATCC (CCL-17; ATCC, Manassas, VA). L1210A is an FR+ human leukemia cancer line that was originally modified by Ratnam *et al.* Karmanos Cancer Center in Detroit MI to express folate receptors (Purdue University, West Lafayette, IN). MM.1S multiple myeloma cells were used as an FR– control and were purchased from ATCC (CRL-2974). All cell lines were cultured in RPMI 1640, folate-deficient media (Gibco 27016–021; ThermoFisher Scientific, Waltham, MA).

Fluorescent Microspheres

We used fluorescent microspheres as a reference standard for evaluating the brightness of labeled cells. “Dragon Green” (DG; DG06M, Bangs Laboratories, Fishers, IN) microspheres are sold in kits of 5 intensities, ranging from 1 (lowest) to 5 (highest). These serve as useful standards for evaluating cell labeling and for comparing data between different instruments, for example, between a flow cytometer (FC) and b-DiFC. Our previous work showed that cells with fluorescent labeling equal to or exceeding DG3 were readily detectable with b-DiFC in SCID mice [33].

Labeling of Cells with FR-Targeted Probes In Vitro

We first tested the labeling of FR+ CTCs with EC-17 in cell culture *in vitro*. Labeling of cultured cells with EC-17 has been well characterized by our team previously [47, 54]. One-milliliter suspensions of 10^6 cells/ml in 2 % FBS in PBS were labeled at an EC-17 concentration of 200 nM (20 μ l of 10 μ M EC-17 stock solution added to 1-ml cell suspensions). Cells were incubated at 37 °C for 60 min, then washed twice with PBS, and resuspended at a concentration of 10^6 cells/ml.

Labeling of Cells in Whole Mouse Blood

We next tested the labeling of FR+ CTCs in whole mouse blood. The blood was drawn from female nude (nu/nu) mice (Charles River Laboratory, Wilmington, MA), which were kept on a folic acid-free diet (TD.00434; Teklad Diet, UK) for at least 2 weeks prior to the study. Drawn blood was stabilized and diluted 1:1 with 1000 units/ml heparin (Sigma Aldrich, Natick, MA). A total of 10^4 CTCs were added to 500 μ l blood aliquots (“spiked”) and then

gently agitated for 30 s to ensure that samples were well mixed. EC-17 was added to cell suspensions to a final concentration of 200 nM (10 μ l of 10 μ M EC-17 stock solution added to 0.5-ml blood samples), and these were incubated for 60 min as above.

To test EC-17 labeling specificity, target (KB, L1210A, or MM.1S) cells were first counter-stained with cell trace far red (CTFR) dye (C34564, Molecular Probes Inc., Eugene, OR) according to the manufacturer's instructions. The rationale here was that well EC17-labeled cells should exhibit double (green and red) positive fluorescence on FC.

Some samples were also co-incubated with 10 μ M of free folic acid to introduce competitive binding. Blood suspensions were analyzed using the blue-green (488-nm laser and 530-nm emission filter) and red (637-nm laser and 647-nm emission filter) channels of an Attune NXT flow cytometer (ThermoFisher). Experiments were repeated at least in duplicate in each case.

DiFC Flow Phantom Experiments In Vitro

As an initial test of the detectability of EC-17-labeled cells with b-DiFC, we used a tissue-simulating flow phantom model (Fig. 1d), as we have in our previous work [32]. Briefly, the flow phantom was a block of optically diffusing high-density polyethylene (HDPE) with similar scatter and absorption properties of biological tissue. A strand of microbore Tygon tubing (TGY-010-C, Small Parts, Inc., Seattle, Washington) was embedded in the phantom block at a depth of 0.75 mm. The tubing was connected to a syringe pump (70–2209, Harvard Apparatus, Holliston, Massachusetts). We prepared suspensions of 10^3 per ml cell suspension of EC-17-labeled L1210A cells per ml, labeled as described in the “Fluorescent Microspheres” section. Cell suspensions were run through the phantom at a rate of 50 μ l/s. We also tested 10^3 spheres/ml suspensions of DG3 reference microspheres at concentrations of 10^3 spheres/ml as a comparison.

DiFC Mouse Experiments In Vivo

All mice were handled in accordance with Northeastern University's Institutional Animal Care and Use Committee (IACUC) policies on animal care. Animal experiments were carried out under Northeastern University IACUC protocol #15–0728R. All experiments and methods were performed with approval from and in accordance with relevant guidelines and regulations of Northeastern University IACUC.

L1210A leukemia cells were used in these experiments since they are known to circulate for extended periods of time when intravenously injected in mice [14]. We first labeled L1210A cells with EC-17 *in vitro* as above. A total of 10^6 labeled cells were suspended in an injection volume of 200 μ L of cell culture media. Female nude mice were anesthetized with isoflurane, and 10^6 cells the cell suspension was injected i.v. *via* the tail vein ($N=3$). We performed b-DiFC on the femoral blood vessel in the mouse leg (Fig. 1e), beginning 10 min after injection of the cells. Because of the smaller size of the mouse leg *versus* the tail, the alignment of both fibers was challenging. Instead, we used single-fiber detection in mice as we have previously [34]. We used a detection threshold of 6 times the standard deviation of the background signal, which was calculated for each minute of the trace. As we discuss, this virtually eliminated detection of false-positive signals due to electronic

noise or motion artifacts. At the end of the b-DiFC scan, approximately 1 ml of blood was harvested (terminal) and EC-17+ cells counted by flow cytometry.

Second, we also performed proof-of-principle testing of EC-17 labeling of FR+ CTCs while in circulation (which we subsequently refer to as “*in vivo* labeling”). Specifically, we injected 10^6 L1210A cells i.v. via the tail vein. Five minutes later, we administered 25 μ g of EC-17 probe in 100 μ l PBS by i.v. injection. The injected probe quantity was based on previously reported work in FR-targeted imaging and CTC labeling in mice *in vivo* and accounts for a significant fast initial excretion and metabolism of the probe [24, 54]. We performed b-DiFC on the mouse hindleg, beginning approximately 40 min after the probe injection (45 min after cell injection) as we discuss in more detail to give sufficient time for the free dye to clear from circulation.

Results

Labeling of FR+ CTCs in Cells In Vitro

We first tested the labeling of FR+ CTCs with EC-17 in cell culture *in vitro*. Fig. 2a shows that FR+ CTC (L1210A and KB) cells were well labeled by co-incubating with 200 nM EC-17 for 60 min, consistent with previously published work [47]. A total of 94.7 % of EC-17-labeled L1210A cells and 94.1 % of labeled KB cells exceed the fluorescence intensity of Dragon Green 3 (DG3) microspheres. As noted above, DG3 is a reference standard that we often use to compare the brightness of labeled cells across instruments; we have shown previously that cells with a brightness exceeding DG3 are readily detectable *in vivo* with b-DiFC [33]. We also verified that increasing the incubation concentration of EC-17 to 2 μ M yielded no increase in cell brightness. In contrast, FR– MM.1S cells displayed negligible uptake, with comparable fluorescence to unlabeled MM.1S, L1210A, and KB cells, illustrating the specificity of the EC-17 probe.

We next tested if EC-17-labeled FR+ CTCs were sufficiently brightly labeled to be detectable with b-DiFC in a tissue-stimulating optical flow phantom (Fig. 1d) [33]. Representative b-DiFC scans of unlabeled L1210A cells (controls), DG3 microspheres, and EC-17-labeled L1210A cells are shown in Figs. 2b–d, respectively. Each fluorescent peak in Figs. 2c, d corresponds to the detection of a fluorescently labeled cell passing through the b-DiFC field of view. The inset panel in Fig. 2c shows the width of an example peak. As summarized in Fig. 2e, the b-DiFC-measured amplitude of EC-17-labeled cells was on average greater than DG3 microspheres, which was consistent with the FC fluorescence data (Fig. 2a). Here, the signal to noise ratio (SNR in dB) is defined as $SNR = 20 \log_{10} (I/\sigma)$, where I is the mean peak amplitude (intensity) and σ is the b-DiFC instrument noise. These *in vitro* data suggest that CTCs well labeled with EC-17 should be detectable in circulation *in vivo* with b-DiFC.

Labeling of FR+ Cells in Whole Mouse Blood

We next tested the labeling of FR+ CTCs in whole mouse blood with EC-17. The blood is a complex suspension of billions of cells per milliliter and therefore presents a more realistic model of *in vivo* labeling where non-specific cell uptake and competitive binding may occur.

The results are summarized in Fig. 3. The green (horizontal axis) and red (vertical axis) autofluorescence of whole mouse blood is shown in Fig. 3a. Addition of EC-17 probe to the blood (only) showed a small amount non-specific uptake of EC-17 (Fig. 3b, green horizontal axis), most likely by macrophages which is consistent with previously reported work [47].

As shown in Figs. 3c and d, blood samples were spiked with L1210A and KB cells that were counter-stained with CTFR (with no EC-17 added). When the EC-17 probe was added to blood suspensions with FR+ CTCs (Fig. 3e), 85.4 % of L1210A CTCs were well labeled with EC-17, meaning that the average brightness of labeled L1210A cells was above non-specific background levels (Fig. 3b). Like-wise, 80.9 % of FR+ KB cells (Fig. 3f) in the blood were well labeled, with mean brightness above background. In contrast, FR– MM.1S cells showed negligible binding (Fig. 3g). The addition of free folic acid to blood samples with L1210A cells prior to the addition of EC-17 (competitive binding) resulted in blockage of subsequent probe binding (Fig. 3h).

The distributions of EC-17 green fluorescence for all the experiments performed in this study are summarized in Fig. 3i. When considering green fluorescence alone, these data show a clear separation between non-specific uptake of blood cells and FR– CTCs with EC-17 (lines 2 and 3) compared with FR+ CTCs labeling by EC-17 (lines 8 and 9).

We also note that the maximum fluorescence labeling of CTCs in the blood was 2.9 times lower than labeling in simple PBS solution *in vitro* (Fig. 2g), which is unsurprising given the complexity of blood compared with PBS. In summary, these *in vitro* blood labeling experiments demonstrated that EC-17 was a promising probe for *in vivo* labeling of FR+ CTCs for b-DiFC.

In Vivo Detection of EC-17-Labeled CTCs with DiFC

We next tested if FR+ CTCs that were well labeled with EC-17 were detectable with b-DiFC in mice *in vivo*. To test this, we first pre-labeled L1210A cells with EC-17 in culture *in vitro* as above and then injected these i.v. *via* the tail vein into nude mice. We performed b-DiFC on the mouse hindleg, approximately above the large femoral blood vessel as in Fig. 1c. Representative b-DiFC data is shown in Fig. 4. Figure 4a shows b-DiFC from a non-injected control mouse. As shown, the background signal was quite stable, with a mean noise standard deviation (σ) of 1.9 mV, and false alarm rate (FAR) of 0.008 counts per minute, when a detection threshold of 6 times σ was used (calculated for each mouse). Figure 4b shows representative b-DiFC data from a mouse injected with pre-labeled L1210A cells. Labeled L1210A cells were clearly detectable with b-DiFC, with a mean signal to noise ratio of 18 dB, although some individual detections exceeded 30 dB. The mean b-DiFC count rate for all 3 mice tested was 0.8 cell counts per minute (48 counts per hour).

Following b-DiFC scanning, we drew 1 ml of blood (terminal) from the mice and counted EC-17+ cells in the blood using fluorescence FC. On average, we counted 13.2 ± 5.7 EC-17-labeled cells per milliliter in blood, where we defined “labeled” as exceeding the green fluorescence intensity of DG3 microspheres. This relatively low number of cells in the blood was expected since it is well established in the literature that most intravenously injected cells are eliminated from circulation on their “first pass” through the vasculature *via*

either the lungs or the liver [55–57], a phenomenon which we have observed in our previous work in mice [32].

Using this cell concentration, we were able to estimate the b-DiFC blood sampling rate from the mouse leg (femoral artery) as follows: the b-DiFC count rate measured during the final 10 min of the scan (just before drawing blood) was 0.6 ± 0.3 counts per minute. Compared with the estimated number of L1210A cells in the blood (13.2 cells/ml), this implies that b-DiFC sampled approximately $0.6 \text{ counts min}^{-1}/13.2 \text{ cells ml}^{-1} = 48 \mu\text{l min}^{-1}$ of blood. A literature search failed to reveal the blood flow rate of the femoral artery in mice for comparison. However, this number is reasonable considering the large size of the blood vessel. It is also lower than our previously reported flow rate ($\sim 300 \mu\text{l/min}$) in the larger ventral caudal artery in the mouse tail [32].

In Vivo Labeling of CTCs with EC-17 and Detection with DiFC

Direct *in vivo* labeling of FR+ CTCs is a more challenging problem, and based on our studies in blood samples, we anticipated that CTC labeling would be significantly less bright than in cell culture *in vitro*. To test this, we first injected a suspension of L1210A cells in mice. Five minutes later, we injected 25 μg of EC-17. The initial bolus of EC-17 resulted in a large non-specific fluorescence background which saturated the b-DiFC detectors. Thus, we waited 40 min after injection for the free dye to sufficiently clear (a duration that was determined empirically) and performed b-DiFC on the mouse hindleg as above. Representative data is shown in Fig. 4c. As shown, we were able to detect a small number of cells in circulation, at an average count rate of 0.1 per minute.

We note that, although there is a rapid “first-pass” removal of many of the injected L1210A cells immediately after injection, based on our measurements, the number of L1210A cells in circulation appeared quite stable following injection. As shown in Fig. 4d, in contrast to our previous work with MM cells [32], the b-DiFC count rate for pre-labeled L1210A cells did not decrease significantly in a 75-min period (85 min after injection). Hence, we interpret the lower measured b-DiFC count rate for *in vivo*-labeled cells (Fig. 4e) as primarily due to significantly lower cell-labeling efficiency by EC-17 in circulation than in cell culture.

Specifically, the histogram of detected peak amplitudes (expressed in mV and dB) *in vivo* are summarized in Fig. 4f. From these data, we can estimate that the lower count rate for *in vivo*-labeled cells could be explained by a reduction of 14-dB labeling brightness. This in turn suggests that cells labeled *in vivo* were approximately 5–6 times lower in fluorescence brightness (labeling) than cells that were pre-labeled in culture. This is reasonable, given that we observed a reduction in EC-17 labeling brightness by a factor of 2.9 when labeling in blood samples compared with PBS (Fig. 3).

However, in aggregate, these data demonstrate the feasibility of detecting CTCs *in vivo* by direct labeling with b-DiFC. Further implications and future steps to increase b-DiFC detection efficiency are discussed in more detail below.

Discussion

We recently developed DiFC, a new technique for detecting and enumerating very rare fluorescently labeled circulating cells in the bloodstream in small animals. Until now, we have only used DiFC with cells that were “pre-labeled” before introduction into the circulatory system, either by using cell lines or transgenic mice genetically modified to express GFP or by labeling with a membrane or cytosol dye [32, 33]. While useful for many mouse models of cancer metastasis, this ultimately restricts the use of DiFC. Therefore, we are interested in developing a receptor-based fluorescence labeling approach [49] since this could greatly extend the pre-clinical utility of DiFC. In addition, because DiFC is inherently scalable to larger limbs and tissues in combination with FDA-approved fluorescent probes [52], this could ultimately open the possibility of the use of DiFC in humans for enumeration of CTCs.

To achieve this, there were two main considerations for use of FR-targeted molecular probes. First, because the blood is a complex mixture of many cell types, the specificity and affinity for CTCs are critical. As shown in Fig. 2, EC-17 demonstrated extremely bright labeling in simple cell cultures. EC-17 also exhibited substantial separation in fluorescence labeling between FR+ CTCs and non-target cells in whole blood (Fig. 3). This is consistent with previously published work with small molecular weight folate receptor-targeted probes [47].

The second major consideration was the general issue of the detectability of fluorescence from a single cell. As noted, DiFC works with diffuse photons in relatively deeply seated (1–2-mm depth) large blood vessels. We previously estimated that DiFC requires labeling with approximately 10^5 fluorescent molecules per cell for detectability [22]. It is well known that blue and green light experiences significantly more attenuation and scatter in biological tissue than red and near-infrared light in general [50] and in the specific case of DiFC may result in loss of approximately 50 % sensitivity [33]. As such, it was unclear if EC-17 receptor-labeled CTCs would be sufficiently bright for detection with b-DiFC, as opposed to GFP-expressing CTCs which are generally very bright [22].

The mouse experiments performed here demonstrated that cells that were labeled in culture prior to injection were readily detectable by b-DiFC in a tissue-mimicking flow phantom model (Fig. 2b–e) and in mice *in vivo* (Fig. 4b) with high SNRs. When the cells and probe were injected separately (“*in vivo* labeling”), we were able to detect several cells but at a count rate of approximately 12.5 % compared with pre-labeled cells. This implies that many cells were labeled below the sensitivity level of b-DiFC and, as we noted, could be explained by a reduction in EC-17 uptake by a factor of 5–6 compared with cell culture. We tried doubling the injected quantity of EC-17 (to 50 μ g) but this simply increased the background signal unacceptably and yielded no appreciable increase in peak SNR. We anticipate that further improvements to the *in vivo* labeling procedures can be achieved in the future.

Related to this, we note that CTC labeling *in vivo* in general will depend on a number of factors that were not fully studied here. For example, the duration between FR-targeted probe administration, the number of receptors per cell, and the probe clearance kinetics

(which may vary based on renal or liver function of the animal) all warrant careful future study and detailed characterization in future work.

Conclusions

In combination, these data provide proof-of-concept for use of small-molecule FR-targeted probes for DiFC. We next plan to pursue DiFC instrument and signal processing improvements to boost detection sensitivity. Moreover, a red or NIR small molecular version of EC-17 such as OTL-38 may improve detectability due to more favorable tissue optics compared with a blue and green light. This work also opens the use of DiFC to other molecular probes that target alternate cell-surface receptors. Finally, because a number of FR receptor-targeted probes (including OTL-38) are in phase 2 and 3 clinical trials [52], this opens the exciting possibility of ultimately using DiFC in humans to enumerate CTCs directly in the bloodstream.

Acknowledgments

The authors thank Dr. Xuefei Tan and Mr. Peter Bartosik for their assistance in performing some DiFC experiments. The EC-17 probe used in this study was provided by On Target Laboratories Inc., West Lafayette, IN.

Funding Information

This study was funded by the National Institutes of Health (R01HL124315; NHLBI).

Data Availability

The datasets generated and analyzed during the current study and the analysis code (written in Matlab) are available from the corresponding author on reasonable request.

References

1. Steeg PS, Theodorescu D (2008) Metastasis: a therapeutic target for cancer. *Nat Clin Pract Oncol* 5:206–219 [PubMed: 18253104]
2. Gupta GP, Massague J (2006) Cancer metastasis: building a framework. *Cell* 127:679–695 [PubMed: 17110329]
3. Pantel K, Speicher MR (2016) The biology of circulating tumor cells. *Oncogene* 35:1216–1224 [PubMed: 26050619]
4. Alix-Panabieres C, Pantel K (2013) Circulating tumor cells: liquid biopsy of cancer. *Clin Chem* 59:110–118 [PubMed: 23014601]
5. de Bono JS, Scher HI, Montgomery RB, Parker C, Miller MC, Tissing H, Doyle GV, Terstappen LWW, Pienta KJ, Raghavan D (2008) Circulating tumor cells predict survival benefit from treatment in metastatic castration-resistant prostate cancer. *Clin Cancer Res* 14:6302–6309 [PubMed: 18829513]
6. Hayes DF, Cristofanilli M, Budd GT et al. (2006) Circulating tumor cells at each follow-up time point during therapy of metastatic breast cancer patients predict progression-free and overall survival. *Clin Cancer Res* 12:4218–4224 [PubMed: 16857794]
7. Liu MC, Shields PG, Warren RD, Cohen P, Wilkinson M, Ottaviano YL, Rao SB, Eng-Wong J, Seillier-Moiseiwitsch F, Noone AM, Isaacs C (2009) Circulating tumor cells: a useful predictor of treatment efficacy in metastatic breast cancer. *J Clin Oncol* 27:5153–5159 [PubMed: 19752342]
8. Cohen SJ, Punt CJ, Iannotti N et al. (2008) Relationship of circulating tumor cells to tumor response, progression-free survival, and overall survival in patients with metastatic colorectal cancer. *J Clin Oncol* 26:3213–3221 [PubMed: 18591556]

9. Toss A, Mu Z, Fernandez S, Cristofanilli M (2014) CTC enumeration and characterization: moving toward personalized medicine. *Ann Transl Med* 2:108 [PubMed: 25489582]
10. Allan AL, Keeney M (2010) Circulating tumor cell analysis: technical and statistical considerations for application to the clinic. *J Oncol* 2010:426218 [PubMed: 20049168]
11. Mader S, Pantel K (2017) Liquid biopsy: current status and future perspectives. *Oncol Res Treat* 40:404–408 [PubMed: 28693023]
12. Shields CW, Reyes CD, Lopez GP (2015) Microfluidic cell sorting: a review of the advances in the separation of cells from debulking to rare cell isolation. *Lab Chip* 15:1230–1249 [PubMed: 25598308]
13. Lalmahomed ZS, Kraan J, Gratama JW, Mostert B, Sleijfer S, Verhoef C (2010) Circulating tumor cells and sample size: the more, the better. *J Clin Oncol* 28:e288–e289 author reply e290 [PubMed: 20439640]
14. Tibbe AG, Miller MC, Terstappen LW (2007) Statistical considerations for enumeration of circulating tumor cells. *Cytometry A* 71:154–162 [PubMed: 17200956]
15. Williams AL, Fitzgerald J, Niedre M (2019) Short-term circulating tumor cell dynamics in mouse xenograft models and implications for liquid biopsy. Preprint available at: bioRxiv 814368. 10.1101/814368
16. Wong KH, Sandlin RD, Carey TR et al. (2016) The role of physical stabilization in whole blood preservation. *Sci Rep* 6:21023 [PubMed: 26876805]
17. Wagar EA, Stankovic AK, Raab S, Nakhleh RE, Walsh MK (2008) Specimen labeling errors: a Q-probes analysis of 147 clinical laboratories. *Arch Pathol Lab Med* 132:1617–1622 [PubMed: 18834220]
18. Delahaye M, Lawrence K, Ward SJ, Hoare M (2015) An ultra scale-down analysis of the recovery by dead-end centrifugation of human cells for therapy. *Biotechnol Bioeng* 112:997–1011 [PubMed: 25545057]
19. Azab AK, Hu J, Quang P, Azab F, Pitsillides C, Awwad R, Thompson B, Maiso P, Sun JD, Hart CP, Roccaro AM, Sacco A, Ngo HT, Lin CP, Kung AL, Carrasco RD, Vanderkerken K, Ghobrial IM (2012) Hypoxia promotes dissemination of multiple myeloma through acquisition of epithelial to mesenchymal transition-like features. *Blood* 119:5782–5794 [PubMed: 22394600]
20. Tuchin VV, Tarnok A, Zharov VP (2011) In vivo flow cytometry: a horizon of opportunities. *Cytometry A* 79:737–745 [PubMed: 21915991]
21. Georgakoudi I, Solban N, Novak J, Rice WL, Wei X, Hasan T, Lin CP (2004) In vivo flow cytometry: a new method for enumerating circulating cancer cells. *Cancer Res* 64:5044–5047 [PubMed: 15289300]
22. Hartmann C, Patil R, Lin CP, Niedre M (2017) Fluorescence detection, enumeration and characterization of single circulating cells in vivo: technology, applications and future prospects. *Phys Med Biol* 63:01TR01
23. Suo Y, Gu Z, Wei X (2019) Advances of in vivo flow cytometry on cancer studies. *Cytometry A*
24. He W, Wang H, Hartmann LC, Cheng JX, Low PS (2007) In vivo quantitation of rare circulating tumor cells by multiphoton intravital flow cytometry. *Proc Natl Acad Sci U S A* 104:11760–11765 [PubMed: 17601776]
25. Novak J, Georgakoudi I, Wei X, Prossin A, Lin CP (2004) In vivo flow cytometer for real-time detection and quantification of circulating cells. *Opt Lett* 29:77–79 [PubMed: 14719666]
26. Zhong CF, Tkaczyk ER, Thomas T, Ye JY, Myc A, Bielinska AU, Cao Z, Majoros I, Keszler B, Baker JR, Norris TB (2008) Quantitative two-photon flow cytometry—in vitro and in vivo. *J Biomed Opt* 13:034008 [PubMed: 18601553]
27. Fan ZC, Yan J, Liu GD, Tan XY, Weng XF, Wu WZ, Zhou J, Wei XB (2012) Real-time monitoring of rare circulating hepatocellular carcinoma cells in an orthotopic model by in vivo flow cytometry assesses resection on metastasis. *Cancer Res* 72:2683–2691 [PubMed: 22454286]
28. Zharov VP, Galanzha EI, Shashkov EV, Kim JW, Khlebtsov NG, Tuchin VV (2007) Photoacoustic flow cytometry: principle and application for real-time detection of circulating single nanoparticles, pathogens, and contrast dyes in vivo. *J Biomed Opt* 12:051503 [PubMed: 17994867]

29. He Y, Wang L, Shi J, Yao J, Li L, Zhang R, Huang CH, Zou J, Wang LV (2016) In vivo label-free photoacoustic flow cytography and on-the-spot laser killing of single circulating melanoma cells. *Sci Rep* 6:39616 [PubMed: 28000788]
30. Galanzha EI, Menyaev YA, Yadem AC, Sarimollaoglu M, Juratli MA, Nedosekin DA, Foster SR, Jamshidi-Parsian A, Siegel ER, Makhoul I, Hutchins LF, Suen JY, Zharov VP (2019) In vivo liquid biopsy using Cytophone platform for photoacoustic detection of circulating tumor cells in patients with melanoma. *Sci Transl Med* 11:eaat5857 [PubMed: 31189720]
31. Zettergren E, Vickers D, Runnels J, Murthy SK, Lin CP, Niedre M (2012) Instrument for fluorescence sensing of circulating cells with diffuse light in mice in vivo. *J Biomed Opt* 17:037001 [PubMed: 22502573]
32. Tan X, Patil R, Bartosik P, Runnels JM, Lin CP, Niedre M (2019) In vivo flow cytometry of extremely rare circulating cells. *Sci Rep* 9:3366 [PubMed: 30833671]
33. Patil R, Tan X, Bartosik P, Detappe A, Runnels JM, Ghobrial I, Lin CP, Niedre M (2019) Fluorescence monitoring of rare circulating tumor cell and cluster dissemination in a multiple myeloma xenograft model in vivo. *J Biomed Opt* 24:1–11
34. Pera V, Tan X, Runnels J, Sardesai N, Lin CP, Niedre M (2017) Diffuse fluorescence fiber probe for in vivo detection of circulating cells. *J Biomed Opt* 22:37004 [PubMed: 28290598]
35. Leamon CP (2008) Folate-targeted drug strategies for the treatment of cancer. *Curr Opin Investig Drugs* 9:1277–1286
36. Parker N, Turk MJ, Westrick E, Lewis JD, Low PS, Leamon CP (2005) Folate receptor expression in carcinomas and normal tissues determined by a quantitative radioligand binding assay. *Anal Biochem* 338:284–293 [PubMed: 15745749]
37. Vlahov IR, Leamon CP (2012) Engineering folate-drug conjugates to target cancer: from chemistry to clinic. *Bioconjug Chem* 23:1357–1369 [PubMed: 22667324]
38. Weitman SD, Lark RH, Coney LR, Fort DW, Frasca V, Zurawski VR Jr, Kamen BA (1992) Distribution of the folate receptor GP38 in normal and malignant cell lines and tissues. *Cancer Res* 52:3396–3401 [PubMed: 1596899]
39. van Dam GM, Themelis G, Crane LM et al. (2011) Intraoperative tumor-specific fluorescence imaging in ovarian cancer by folate receptor-alpha targeting: first in-human results. *Nat Med* 17:1315–1319 [PubMed: 21926976]
40. O'Shannessy DJ, Somers EB, Smale R, Fu YS (2013) Expression of folate receptor-alpha (FRA) in gynecologic malignancies and its relationship to the tumor type. *Int J Gynecol Pathol* 32:258–268 [PubMed: 23518909]
41. Vergote IB, Marth C, Coleman RL (2015) Role of the folate receptor in ovarian cancer treatment: evidence, mechanism, and clinical implications. *Cancer Metastasis Rev* 34:41–52 [PubMed: 25564455]
42. Toffoli G, Cernigoi C, Russo A, Gallo A, Bagnoli M, Boiocchi M (1997) Overexpression of folate binding protein in ovarian cancers. *Int J Cancer* 74:193–198 [PubMed: 9133455]
43. Tummers QR, Hoogstins CE, Gaarenstroom KN, de Kroon CD, van Poelgeest M, Vuyk J, Bosse T, Smit VT, van de Velde C, Cohen AF, Low PS, Burggraaf J, Vahrmeijer AL (2016) Intraoperative imaging of folate receptor alpha positive ovarian and breast cancer using the tumor specific agent EC17. *Oncotarget* 7:32144–32155 [PubMed: 27014973]
44. O'Shannessy DJ, Somers EB, Maltzman J, Smale R, Fu YS (2012) Folate receptor alpha (FRA) expression in breast cancer: identification of a new molecular subtype and association with triple negative disease. *Springerplus* 1:22 [PubMed: 23961352]
45. Shi H, Guo J, Li C, Wang Z (2015) A current review of folate receptor alpha as a potential tumor target in non-small-cell lung cancer. *Drug Des Devel Ther* 9:4989–4996
46. O'Shannessy DJ, Yu G, Smale R, Fu YS, Singhal S, Thiel RP, Somers EB, Vachani A (2012) Folate receptor alpha expression in lung cancer: diagnostic and prognostic significance. *Oncotarget* 3:414–425 [PubMed: 22547449]
47. He W, Kularatne SA, Kalli KR, Prendergast FG, Amato RJ, Klee GG, Hartmann LC, Low PS (2008) Quantitation of circulating tumor cells in blood samples from ovarian and prostate cancer patients using tumor-specific fluorescent ligands. *Int J Cancer* 123:1968–1973 [PubMed: 18661519]

48. Forster MD, Ormerod MG, Agarwal R, Kaye SB, Jackman AL (2007) Flow cytometric method for determining folate receptor expression on ovarian carcinoma cells. *Cytometry A* 71:945–950 [PubMed: 17712798]
49. Pitsillides CM, Runnels JM, Spencer JA, Zhi L, Wu MX, Lin CP (2011) Cell labeling approaches for fluorescence-based in vivo flow cytometry. *Cytometry A* 79:758–765 [PubMed: 21905206]
50. Jacques SL (2013) Optical properties of biological tissues: a review. *Phys Med Biol* 58:R37–R61 [PubMed: 23666068]
51. Predina JD, Newton AD, Keating J, Dunbar A, Connolly C, Baldassari M, Mizelle J, Xia L, Deshpande C, Kucharczuk J, Low PS, Singhal S (2018) A phase I clinical trial of targeted intraoperative molecular imaging for pulmonary adenocarcinomas. *Ann Thorac Surg* 105:901–908 [PubMed: 29397932]
52. Lee JYK, Cho SS, Stummer W, Tanyi JL, Vahrmeijer AL, Rosenthal E, Smith B, Henderson E, Roberts DW, Lee A, Hadjipanayis CG, Bruce JN, Newman JG, Singhal S (2019) Review of clinical trials in intraoperative molecular imaging during cancer surgery. *J Biomed Opt* 24:1–8
53. De Jesus E, Keating JJ, Kularatne SA et al. (2015) Comparison of Folate receptor targeted optical contrast agents for intraoperative molecular imaging. *Int J Mol Imaging* 2015:469047 [PubMed: 26491562]
54. Paulos CM, Reddy JA, Leamon CP, Turk MJ, Low PS (2004) Ligand binding and kinetics of folate receptor recycling in vivo: impact on receptor-mediated drug delivery. *Mol Pharmacol* 66:1406–1414 [PubMed: 15371560]
55. Pond SM, Tozer TN (1984) First-pass elimination. Basic concepts and clinical consequences. *Clin Pharmacokinet* 9:1–25
56. Fischer UM, Harting MT, Jimenez F, Monzon-Posadas WO, Xue H, Savitz SI, Laine GA, Cox CS Jr (2009) Pulmonary passage is a major obstacle for intravenous stem cell delivery: the pulmonary first-pass effect. *Stem Cells Dev* 18:683–692 [PubMed: 19099374]
57. Mizuno N, Kato Y, Izumi Y, Irimura T, Sugiyama Y (1998) Importance of hepatic first-pass removal in metastasis of colon carcinoma cells. *J Hepatol* 28:865–877 [PubMed: 9625323]

Fig. 1.
a b-DiFC instrument schematic, reproduced with permission from Patil *et al.* [33]. See the text for details. **b** Photograph of the b-DiFC cart-mounted system, reproduced with permission from Patil *et al.* [33]. The dual-fiber DiFC probe head (red arrows) is placed on the surface of the sample, in this case, either **c** the hindleg of a mouse (femoral artery) or **d** a tissue-mimicking flow phantom. This permits the detection and enumeration of fluorescently labeled circulating cells.

Fig. 2.

a Green fluorescence flow cytometry (FC) analysis of EC-17 labeling of FR+ L1210A and KB cells, and FR- MM.1S cells in culture *in vitro* are shown. EC-17 had a high affinity for L1210A and KB cells, whereas little binding to MM.1s cells was observed above background. Representative b-DiFC measurements from **b** unlabeled (control) L1210A cells in culture in a flow phantom, **c** DG3 reference fluorescent microspheres, and **d** EC-17 labeled L1210A cells, which were readily detectable with b-DiFC. **e** Summary of the mean peak amplitudes measured with b-DiFC in each case is shown.

Fig. 3. Labeling of FR+ CTCs with EC-17 in whole mouse blood. **a** Red and green autofluorescence of blood (only). **b** Addition of EC-17 by blood (only), showing minimal binding or uptake by non-specific cells. **c** L1210A and **d** KB cells were counter-stained with cell trace far red (CTFR) and added to the blood. Significant specific labeling of **e** L1210A and **f** KB cells was observed when EC-17 was added to blood solutions spiked with FR+ CTCs. **g** Addition of free folic acid (prior to addition of EC-17) competitively blocked binding of EC-17 to L1210 cells in the blood. **h** FR- MM.1s cells showed negligible EC-17 uptake in the blood. **i** Summary of green fluorescence FC measurements for all experiments performed is shown.

Fig. 4.

a Representative b-DiFC measurement from the hindleg of an un-injected control mouse. **b** L1210A cells that were pre-labeled in culture with EC-17 were readily detectable in mice *in vivo*. **c** Unlabeled L1210A cells were injected, followed by EC-17 (5 min later), showing proof-of-concept of *in vivo* labeling of CTCs. b-DiFC was performed 40 min after injection of the probe. **d** The b-DiFC-count rate from pre-labeled L1210A cells indicating minimal clearance from circulation on a 75-min timescale (85 min post-cell injection). **e** The average b-DiFC-measured false alarm rate, pre-labeled, and *in vivo*-labeled DiFC count rates. **f** Histogram of b-DiFC-measured peak amplitudes. In combination, these data (**e**, **f**) suggest that the lower measured count rate was due to a factor of 5–6 reduced cell labeling for *in vivo* labeling of cells compared with pre-labeled cells in culture.