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Perfusion-based fluorescence imaging method delineates diverse organs and identifies multifocal tumors using generic near infrared molecular probes

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Abstract

Rapid detection of multifocal cancer without the use of complex imaging schemes will improve treatment outcomes. In this study, dynamic fluorescence imaging was used to harness differences in the perfusion kinetics of near infrared fluorescent dyes to visualize structural characteristics of different tissues. Using the hydrophobic non-tumor-selective near infrared dye cypate, and the hydrophilic dye LS288, a high tumor-to-background contrast was achieved, allowing the delineation of diverse tissue types while maintaining short imaging times. By clustering tissue types with similar perfusion properties, the dynamic fluorescence imaging method identified secondary tumor locations when only the primary tumor position was known, with a respective sensitivity and specificity of 0.97 and 0.75 for cypate, and 0.85 and 0.81 for LS288. Histological analysis suggests that the vasculature in the connective tissue that directly surrounds the tumor was a major factor for tumor identification via perfusion imaging. Although the hydrophobic dye showed higher specificity than the hydrophilic probe, use of other dyes with different physical and biological properties could further improve the accuracy of the dynamic imaging platform to identify multifocal tumors for potential use in real-time intraoperative procedures.

Graphical Abstract

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Some tumors have multiple nodules in different positions in the same organ, making their detection and surgical removal challenging. In this study, generic near infrared fluorescence dyes and dynamic contrast enhanced (DyCE) imaging were used to identify different tissue structures and organs within minutes post injection of the dye. Instead of selecting the entire organ within a region of interest to obtain the basis functions, a seed-based approach was used, where only a small number of pixels were needed to determine the proper basis function. In addition to identifying different structures with a single dye, the DyCE imaging detected satellite tumors once the major tumor was known based on the similarity in their perfusion properties. Data analysis showed that cancer detection was primarily driven by the tumor vasculature. Because primary (major) tumors are readily identifiable during surgery, this work highlights the possibility of rapidly visualizing satellite tumors based on the perfusion properties of primary cancer, thereby facilitating complete tumor removal and improving surgical outcomes.

Keywords

cancer; optical; near-infrared; basis function; molecular probe

1. Introduction

Detection of multifocal tumors remains a challenge in the clinic due to several factors, including the difficulty in identifying the multiple cancer foci in the same organ [1]. Currently, these tumors are mapped with conventional imaging methods such as computed tomography and magnetic resonance imaging [2]. Breast-conserving surgery is now becoming an acceptable treatment method for these patients [3], accentuating the need to accurately detect and remove the multiple foci in real time. Unlike conventional imaging methods, fluorescence imaging has emerged as a viable alternative for image-guided surgery because it does not require the use of ionizing radiation and can display images of tumors in real time [4–8]. To improve the accuracy of detecting tumors, most fluorescence imaging techniques rely on the selective accumulation of a tumor-targeting fluorescent molecular probe in the lesions. A lag time post injection of the molecular probe is needed before imaging to allow the clearance of the imaging agent from the surrounding healthy tissue. This interval could be as short as 1 hour or as long as 96 hours for small molecular or antibody-based imaging agents, respectively, which could interfere with routine surgical planning. An alternative approach that uses activatable molecular probes can selectively enhance fluorescence in tumors, thus facilitating tumor detection without waiting for the clearance of the agent from surrounding tissue [9–11]. The accuracy of this approach relies on the selective expression of the activating agent such as enzymes and the retention of the imaging signal in tumors post activation.

Regardless of the approach, conventional fluorescence techniques lack structural information that is useful for assessing highlighted tumors relative to other tissues. Moreover, none of the tumor-targeting fluorescent molecular probes have received regulatory approval for routine use in humans. In contrast, generic fluorescent agents such as indocyanine green, fluorescein, and methylene blue are approved for use in humans. Several studies have shown that these agents can be repurposed for cancer imaging in the clinic [12]. As a result, a method that could harness the perfusion properties of these generic (non-tumor targeted) fluorescent dyes to delineate different tissue types could accelerate the clinical translation of fluorescence imaging method for the detection of multifocal lesions.

Although widely used in cancer imaging, static fluorescence imaging methods are not capable of simultaneously generating adequate fluorescence contrast for delineating multiple structures of different tissue types using the same molecular probe. Previous reports have shown that dynamic contrast enhanced (DyCE) small animal imaging can utilize the differential interactions of a generic (non-tumor targeting) fluorescent dye with tissue to delineate multiple structures [13]. The method relies on the temporal changes of a fluorescent molecular probe *in vivo* based on perfusion, circulation, dye kinetics, molecular interactions, and a number of other parameters. By generating spatiotemporal fluorescence images in a short amount of time, DyCE imaging can achieve different tissue contrasts without using tumor-targeted or activatable molecular probes [14]. DyCE imaging has been used to investigate many applications, ranging from modeling vascular disease [15] and identifying brain tumor hemisphere [16] to monitoring antiangiogenic therapy [17]. A recent study extended the perfusion based imaging approach from preclinical to clinical uses, highlighting the translatability of the approach [18].

In this study, we developed an animal model of multifocal breast cancer by implanting tumors of the same origin in different mammary fat pad locations. Using this cancer model, we tested the hypothesis that the similarity of the perfusion properties of tumors of the same origin located in different regions in the body will allow their detection based on dynamic fluorescence imaging. Simultaneous imaging of the tumors and surrounding non-tumor structures was executed, and the sensitivity and specificity of perfusion based imaging were evaluated. The results demonstrate that the method is capable of identifying multifocal tumors when only the primary tumor location was known.

2. Materials and Methods

2.1 Animal model development

All studies were conducted in compliance with the Washington University Animal Welfare Committee's requirements for the care and use of laboratory animals in research. Female athymic nude mice were used for all imaging studies. The HT1080 (human fibrosarcoma) mouse model was created by injecting 5×10^6 cells into the flank of the mouse. The orthotopic 4T1-Luc (murine mammary cancer) mouse models were produced by injecting 0.1×10^6 cells into the mammary fat pads bilaterally. The HT1080 tumors grew for 3 weeks prior to imaging, and the 4T1-Luc tumors grew for 2 weeks prior to imaging. Imaging was conducted under anesthesia using 2.5% isoflurane in oxygen. Animals were euthanized after imaging and the 4T1-Luc tumors were harvested for histology.

2.2 Perfusion imaging studies

Perfusion imaging studies were conducted following the general setup as previously reported [14], with a dorsal or ventral view and both orthogonal side views visible. A cooled CCD camera (ORCA-ER C4742–80, Hamamatsu, JP), with an 830 ± 10 nm BP filter (Thorlabs), was used for detection. Imaging was conducted at a frame rate of 1.3 frames/sec, with a 400 msec exposure time, and the f-stop set to 2.0. Excitation was achieved using a wide-field LED illumination source consisting of 16 mounted IR high power single chip LEDs (Roithner LaserTechnik, AU). Peak illumination was set at 760 nm, with a power output of 0.6 mW/cm^2 .

The NIR fluorescent dyes cypate and LS288 (Figure 1c) were synthesized as previously reported [19, 20]. Cypate and LS288 possess hydrophobic and hydrophilic properties, respectively [21]. The samples were prepared by dissolving trace amounts of the dyes into 3 mL of DMSO in $1 \times 1 \text{ cm}^2$ quartz cuvettes at 25°C . The emission was measured at the excitation of 720 nm using a 15.24 cm integrating sphere Quanta-phi fiber-optically connected to Horiba fluorimeter equipped with a deep-cooled diode array detector. The emission spectra (Figure 1f) were corrected for the instrument response generated according to the instrument specification prior the measurements. For imaging studies, each dye was mixed to a concentration of $100 \mu\text{M}$ in PBS with 20% DMSO, and $100 \mu\text{L}$ of total volume was injected into the mouse using tail vein catheters. The catheters were constructed of 0.015 in. ID $\times$ 0.033 in. OD polyurethane tubing (PU-033, SAI Infusion Technologies) and 29 gauge needles. Because of the extended length of the catheters, we accounted for the dead-space within the catheter ($50 \mu\text{L}$) when the total volume of dye was calculated.

2.3 Bioluminescence imaging

Bioluminescence imaging was conducted on the 4T1-Luc xenograft mice expressing luciferase to confirm bilateral tumor location. These mice received an intraperitoneal injection of 150 mg/kg D-luciferin in PBS (Gold Biotechnology). Mice were then imaged at 10 minutes post injection under isoflurane anesthesia with an IVIS Lumina imaging system (PerkinElmer, Living Image 3.2, 10-second exposures, bin8, FOV 12.5 cm, f/stop1, open filter). The total photon flux (photons/sec) was measured from software-defined contour regions of interest (ROIs) over the tumors using Living Image 2.6.

2.3 Immunohistochemistry

Frozen tissue slides ($5 \mu\text{m}$) were fixed by immersing slides in acetone for 5 minutes and air dried. Slides were rehydrated in DPBS and treated with dual endogenous enzyme block (Dako), TBS/0.1% Tween-20 (TBST) wash buffer, and 10 minutes of serum-free protein block (Dako). Rabbit anti-mouse CD31 antibody (D8V9E, Cell Signaling Technology) was used as primary antibody at 1:100 dilution. Following primary antibody incubation, slides were extensively washed in TBST. Anti-Rabbit EnVision+ System-HRP (Dako) was used as the secondary antibody, followed by Liquid DAB+ ($3,3'$ -Diaminobenzidine) Substrate system (Dako), both according to the manufacturer's protocol. Nuclear hematoxylin counterstain was applied, followed by dehydration through 70% ethanol, 95% ethanol, 100% ethanol, and xylene. Slides were mounted with Cytoseal XYL (Thermo Scientific).

3. Results

3.1 Perfusion algorithm development

We modified the DyCE image analysis method [14] to meet the needs of the current study. Images were obtained in grayscale in individual TIFF files over the imaging duration. The image files were read into MATLAB (Mathworks, Inc.), where they were combined into a stack of images. A single image was used to select the center point of the regions of interest (ROIs) to obtain the basis functions. These center points were termed seeds and were selected by the operator. Because the goal of the study was to allow the method to determine similar pixels rather than have the user define the pixels to identify, a small ROI of 5×5 pixels was selected for analysis. The average fluorescence intensity value of the ROI was calculated for each time point, and this time course of values was the representative basis function for that tissue type. This process was repeated until each tissue type of interest was identified in the field of view.

Once each basis function was identified, all of the pixels in the field of view were analyzed using the nonnegative least-squares fit function in MATLAB (`lsqnonneg`). Each pixel was fit to all of the basis functions, and the degree of the fit for each basis function determined the extent of the color represented in each pixel. To generate the image, each pixel was assigned a color with the normalized percentage of the color shading, matching the degree of fit to each basis function (more opaque coloring for a higher degree of fit). The DyCE imaging method additionally used normalized and background-subtracted basis functions for the pixel time courses. However, we did not see an improvement in our results by using these additional mathematic steps. As such, we utilized the raw fluorescence intensity for the analysis. The perfusion based algorithm was able to generate pixel based images that represented both spatial and temporal data.

3.2 In vivo perfusion imaging

Previous studies have determined the emission spectra of these dyes using photomultiplier tubes that are not highly sensitive in the NIR wavelengths above 750 nm [19–21]. Using a NIR light-sensitive CCD camera, which is employed for in vivo imaging, we found that the corrected emission maxima for LS288 and cypate were 820 and 826 nm, respectively, when excited at 720 nm (Figure 1f). Therefore, we selected 760 nm excitation and 820 – 840 nm bypass filter to minimize detecting reflected light and capture sufficient fluorescent signal for both dyes. To determine the appropriate dye for perfusion based tumor identification, we used the HT1080 flank tumor model as our test case. We started with cypate, a hydrophobic NIR dye that has been previously described [22, 23]. Figure 1a shows the *in vivo* imaging setup used to capture the dorsal and side views of the animal, with Figure 1b showing the seed locations used to generate organ-specific basis functions. Figure 1d shows the pixel fluorescence intensities for the basis functions for each tissue type corresponding to the seeds selected. The *in vivo* kinetics of cypate were consistent with previous studies, displaying a strong liver localization at short time points [24]. As a result, other tissues exhibited decreasing fluorescence over time with decreased contrast between tissue types. Figure 1e shows the visual representation of the non-negative least-squares fitting of each pixel to the appropriate basis function. Pixels with similar kinetics to the basis function were

represented with the same color. Differentiation between tissue types was apparent due to their differing characteristic perfusion kinetics.

We next injected the hydrophilic NIR fluorescent dye, (Figure 1c,f). Previous work has shown that the hydrophilic nature of LS288 contributes to increased tissue permeation as compared to cypate [21]. Hydrophobic dyes, such as cypate, have a higher binding affinity to blood proteins such as albumin and are therefore more readily removed from the circulation by the liver. Figure 1g shows the LS288 basis functions and Figure 1h shows the visual representation of the basis function curve fits. In the case of the LS288 basis functions, each of the different tissues exhibited increased differentiation as time progressed as opposed to cypate that exhibited decreased differentiation over time (with the exception of the liver). We determined that the increased differentiation between tissues with increasing time was advantageous for our method, favoring the use of LS288 in subsequent experiments.

3.3 Method optimization

Next, we optimized the perfusion method to reduce imaging artifacts from motion and external light. To capture early time points, tail-vein injection of the dye was performed via a catheter after placing the mouse in an enclosed black box. This setup allowed us to inject the dye into the tail vein without the need to open the box and expose the field of view to external light. As expected, the shapes of the basis function curves for the kidney, liver, brain, and tail (Figure 1i) were similar to the open box imaging. However, the skin basis function curve had a lower signal relative to other tissues. In this study, we used the term skin to represent any tissue that did not have recognizable organs or structures beneath it. This feature could include a combination of skin, subcutaneous tissue, and fat. Because skin is highly reflective, any uncontrolled external light may impact the result more so than the other organs. Figure 1j shows the visual representation of the non-negative least-squares fitting of each pixel to the appropriate basis function.

A region on the flank without tumor was selected to ensure the method was not automatically associating flank tissue with tumor. The expected perfusion profile would be for skin alone. Figure 1k shows that the least-squares fit associated with the hypothetical tumor selection was consistent with the skin as expected. This observation increased confidence that the method was assigning pixels to a tumor basis function only when a tumor was present. We also imaged the ventral side of the mouse to visualize additional organs (Figure 1l and 1m).

3.4 Multifocal tumor identification

Once we determined that using a tail vein catheter and eliminating light variability produced superior results, we investigated if the method could identify multifocal tumors. We used a 4T1-Luc orthotopic model, with bilateral mammary fat pad tumors. One mouse had tumors visible from the dorsal side, and the other had tumors visible from the ventral side (Figure 2a). When the mice were imaged using planar fluorescence intensity alone, without considering perfusion time, there was minimal contrast outlining the various tissue types and organs (Figure 2b). We confirmed the location of the tumor cells via bioluminescence imaging (BLI) (Figure 2c). The BLI image analysis allowed us to identify the larger tumor

and test the ability of the method to locate a secondary tumor when the primary tumor location was known. The tumor with the higher BLI signal was considered the primary tumor, and the tumor with the lower BLI signal was considered the secondary tumor. Primary and secondary tumors were defined in this study to simulate the clinical scenario where the primary tumor location is known but not the smaller multifocal secondary lesions. For tumor visualization using perfusion imaging, the primary tumor was selected on one side of the mouse, and all other tissues with similar kinetics were identified by the method. In both mice, the secondary tumor on the contralateral side of the mouse was identified when only the primary tumor was selected (Figure 2d). Figure 2e and Figure 2f show the seed locations selected for each organ in the dorsal and ventral views, respectively.

The perfusion method largely identified tumors in similar locations as the BLI, with the exception of regions of the dorsal view that showed a false positive on the back, and the ventral view that identified an area on the periphery of the secondary tumor. Because this method is based on perfusion kinetics, it is possible that the regions identified as a tumor had similar vascular kinetics, although they did not contain the actual tumor cells. Figure 3a shows two large blood vessels feeding the surgically reflected primary tumor. A combination of the large vessels and thin overlying tumor tissue around the periphery may explain the propensity of the method for increased localization of the tumor to the periphery of the BLI tumor locations.

3.5 Method analysis

To examine the ability of the method to detect the presence of a tumor, we investigated the tumor basis functions for each of the three LS288 injections as well as the cypate injection (Figure 3b). We found that the LS288 injections exhibited similar tumor perfusion kinetics and that these basis functions differed from that of cypate. Because the perfusion kinetics were similar for a given dye in tumor tissue, this method may be amenable to using a predictive basis function curve to identify tumors. A predictive standard curve may allow for using the method without *a priori* information from the image itself. Histological evaluation of the tissue using CD-31 staining showed that there was increased vasculature in the tumor as compared to the surrounding muscle (Figure 3b and 3c). The connective tissue directly surrounding the tumor showed the greatest degree of vascular density, consistent with our gross pathological findings (Figure 3a).

To further investigate the sensitivity and specificity of the method, we developed a mask to represent the tumor region and considered this as our ground truth (Figure 4). The mask was generated via visual inspection of the fluorescence image, with confirmation of the tumor locations using the BLI. The perfusion results for the tumors were compared to the mask (Figure 5a). The sensitivity and specificity were calculated by comparing the method result to the mask and determining the true and false positive rates on a pixel-by-pixel basis. The sensitivity and specificity of the method, based on an entire imaging run, were plotted for the cypate injection and the three LS288 injections (Figure 5b and 5c). The sensitivity and specificity of the method were plotted for each run, demonstrating that the method consistently performed better than the random guess line (Figure 5d).

The imaging time post injection used for image analysis could alter the accuracy of tumor detection. We determined this effect by using the mouse imaged from the ventral view and plotting the sensitivity and specificity as we varied the length of video analyzed (Figure 4e). In the case of LS288, the sensitivity was high at short imaging times (< 13 sec post injection), then fell for intermediate times (24 – 90 sec post injection), and increased again at longer imaging times (90 – 120 sec post injection). The specificity increased with increased imaging time until it reached an asymptote at around 24 seconds post injection. A receiver operating characteristic curve was created to simultaneously consider sensitivity and specificity, and three distinct groupings were apparent (Figure 5f). Group A had high sensitivity and was imaged for the least amount of time. However, this group also had the highest false positive rate. Group B balanced the sensitivity and specificity, and consisted primarily of longer imaging times. Interestingly, Group C, which consisted of the intermediate imaging times, had the lowest sensitivity. The result suggests that there is a period post injection that the dye kinetics are similar in different tissues, thus limiting differentiation. Figure 5g shows the basis functions for both the tumor and skin. Sensitivity at early time points may be driven by the slope difference between the curves, with tumors having a more rapid early uptake of dye. Specificity appears to be driven by the magnitude of the difference between the curves.

4. Discussion

Identifying multifocal tumors using perfusion could allow a clinician to rapidly check for additional pathology at the point of care. We selected an orthotopic breast cancer model to test our hypothesis for secondary tumor identification because breast cancers can often be multifocal in nature [25, 26]. There is a possibility of Positive tumor margins are present in 20–70% of breast cancer surgeries, with multifocal tumors increasing the risk of margin positivity [27, 28]. Previous studies have shown that intraoperative evaluation during tumor resection procedures can reduce the risk of leaving residual disease after surgery [29]. Optical imaging allows for real-time imaging and tumor foci identification, making it useful for intraoperative guidance and tissue evaluation [4]. A surgeon could use fluorescence guidance to reduce the probability of leaving an unidentified residual tumor. Fluorescence imaging has already been employed intraoperatively to identify sentinel lymph nodes and visualize primary tumors [5, 6]. Our perfusion based imaging approach, when combined with intraoperative imaging, could reduce the chance of leaving a small tumor loci after a surgical procedure.

Previous work suggested that using a basis function approach for data analysis could increase the sensitivity and specificity for structural analysis as compared to a principal component analysis approach [13, 14]. This approach was successfully used to identify different organs [14, 16]. Depending on the intended application, other features of perfusion imaging could be more important. In breast conserving surgery, for example, the organ is known, but approaches that will reduce the complexity in rapidly identifying multiple tumor nodules are highly needed. As a result, we explored the use of perfusion imaging to identify multifocal tumor pathology while still observing non-tumor structural information.

We demonstrated our method in both a single and a bilateral tumor model. In the single tumor model, a 5×5 pixel area was selected, and all of the other similar pixels were clustered and represented as such. Previous work selected the entire organ within an ROI to obtain the basis functions [14]. Our approach utilized a seed-based approach where only a small number of pixels are needed to determine the proper basis function. Because primary (major) tumors are readily identifiable during surgery, our work highlights the possibility of rapidly visualizing satellite tumors based on the perfusion properties of the primary tumor. However, the seed-based approach introduces inherent variability in the method. Further optimization of the method would allow for an automated approach to determining the proper seed locations for generating the most representative basis functions.

We chose NIR fluorescent dyes, as opposed to visible dyes, to maximize light penetration in tissue for sub-surface structural delineation. Harnessing the versatility of the *in vivo* kinetics of NIR dyes to highlight different structures could allow the perfusion method to be tailored to obtain optimal contrast based on the structures of interest, while still achieving adequate contrast and maintaining short imaging times. Although static fluorescence imaging of cancer using tumor-targeted fluorescent dyes can identify multifocal tumor nodules, the process often takes a number of hours for the dye to accumulate in the tumor and clear from surrounding non-tumor tissue, a delay that can be avoided by using the perfusion imaging strategy. Many simple NIR fluorescent imaging dyes with distinct physical and biological properties are available today. Screening of these dyes could be used to establish the best structural features for cancer imaging using perfusion method. From these dyes, it may be possible to identify a molecular probe that can maintain differences in tissue uptake for several hours. For rapid clinical translation, repurposing of NIR fluorescent dyes such as indocyanine green could be advantageous. Not only could this approach avoid the high cost of developing a new tumor-targeted contrast agent, it will also accelerate the adoption of fluorescence-based perfusion imaging in the clinic.

Tumors often exhibit increased angiogenesis, with larger blood vessels, as compared to healthy tissue [30, 31], making a perfusion based approach a useful technique for tumor detection. Because tumors have different vascularity compared to other tissues and organs, the introduction of a fluorescent dye gives insight into the tumor perfusion kinetics, which may improve our understanding of tumor physiology. For example, HT1080 tumors had similar perfusion properties as the skin when using the hydrophobic cypate dye. When the hydrophilic LS288 dye was used, the same tumor displayed similar profiles as the major excretion organs (liver and kidney) rather than the skin (Figure 1c and 1e). One could identify tumor characteristics by comparing the tumor basis functions to those of other organs. When imaging the 4T1-Luc breast tumors, the increased sensitivity of LS288 at the early and late time points could be attributed to the hydrophilic nature of the dye, which minimizes binding to blood proteins and enhances rapid extravasation and perfusion of all tissue. At early time points, the method did not clearly differentiate tumor pixels and those corresponding to surrounding structures, as evidenced by the high sensitivity and low specificity. At late time points, the LS288 basis function curves were sufficiently different to allow the separation of the tumor from non-tumor tissues. The driving characteristic of differentiation of the perfusion curves at late time points was the magnitude rather than the initial slope of the basis curves. This mechanism is intuitive as a hydrophilic dye will

experience more passes through the systemic circulation than a hydrophobic dye that is rapidly sequestered by the liver. As a result, the tumor tissue that was highly vascularized experienced an increase in the fluorescence signal over time and was identified with both a higher sensitivity and specificity than at earlier time points. Immunohistochemical analysis supported the major role of increased vasculature in the tumor region as compared to the surrounding tissue (Figure 3). By using multiple tumor models, we showed that using the same dye with our method produced similar tumor identification results, regardless of the tumor type. This result suggests that our method is more sensitive to the tumor vasculature than differences in the individual cellular characteristics. Tumors with different sizes can have different perfusion parameters, which can compromise our ability to cluster similar lesions and organs on the basis of their perfusion profiles. Evaluation of how tumor size, vascular density, and organ location affects the identification of multifocal tumors will further enhance the impact of this approach in cancer detection.

5. Conclusion

We have demonstrated a perfusion based imaging method for tumor identification *in vivo* using a basis function approach with least-squares fitting to analyze the perfusion images. Our perfusion based algorithm was able to generate pixel based images that represented both spatial and temporal information. Evaluation of two NIR fluorescent dyes with distinct aqueous solubility and binding to blood proteins show that the vascular retention caused by blood protein-binding of cypate and longer retention in diverse tissue types caused by the rapid extravasation of LS288 modulated the temporal differentiation of tumor from surrounding non-tumor structures. The DyCE imaging revealed an interplay between data acquisition time and the accuracy of tumor detection. Whereas the sensitivity of the method was low at intermediate time points, early and late time point measurements post injection of the dyes exhibited a higher sensitivity for LS288. The results suggest that the initial extravasation and subsequent intravasation of the dye were dependent on the tissue type. At equilibrium, represented by the intermediate time (24 – 90 sec) post injection, the dynamics of dye distribution were less dependent on the tissue types. The accuracy of using our algorithm to predict satellite tumors once the primary tumor is known was validated *in vivo* by bioluminescence imaging. While the sensitivity of tumor detection is probably driven by the slope difference between the curves at early time points, the specificity was dominated by the magnitude of the difference between the curves. In this study, we used a seed-based approach to select a small number of pixels for basis function determination, which introduces variability in the method. Developing an automated approach to determining the proper seed locations for generating the most representative basis functions will improve the throughput of image analysis and accuracy of the method.

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Figure 1.

In vivo perfusion imaging for cypate and LS288. (a) Imaging setup for perfusion imaging. Mirrors mounted at 45 degree angles on both sides of the mouse for additional views. Fluorescence image of cypate injected via the tail vein. (b) Seed locations selected for organ-specific basis functions. (c) Chemical structures of cypate and LS288. (d) Fluorescence intensity vs. time for cypate (organ specific basis functions). (e) Visual representation of the pixel least-squares fit to the cypate basis functions. Each pixel is represented by the corresponding basis function color. The tumor located on the left flank is indicated by the arrow. (f) Corrected fluorescence spectra of cypate and LS288 in dimethylsulfoxide at 720 nm excitation using a CCD camera (g) Fluorescence intensity vs. time for LS288. (h) Visual representation of the pixel least-squares fit to the LS288 basis functions. (i) Dorsal view LS288 basis functions obtained when imaging in an enclosed box without external light. The skin signal was lower than in (g) when no external light was present. (j) Dorsal view fit to the LS288 basis functions. (k) Area on the flank selected (asterisks) to confirm that the method does not give a tumor false positive when no tumor is present. (l) Ventral view LS288 basis functions obtained when imaging in an enclosed box without external light. (m) Ventral view fit to the LS288 basis functions.

Figure 2.

Multifocal tumor identification. (a) Bright field image of a 4T1-Luc orthotopic mouse model with bilateral tumors. Mouse from a dorsal view (top row), and a different mouse from a ventral view (bottom row). (b) Static fluorescence image of LS288 showing limited organ contrast. (c) BLI of tumor signal localizing the tumors and identifying the primary and secondary tumor based on the BLI signal. (d) Visual representation of the pixel least-squares fit to the basis functions. Tumor locations indicated by arrows, and primary tumor location selected for the basis function indicated by the asterisks. The images in (e) and (f) show the seed locations selected for the individual basis functions.

Figure 3.

Perfusion kinetics and tumor vasculature evaluation. (a) Image showing the primary tumor from the ventral view with two large vessels feeding the tumor located on the periphery (arrows). (b) Tumor perfusion time courses for cypate and LS288 across different injection runs. (c) CD-31 staining (brown) of tumor showing increased vascularity in the connective tissue (CT) directly surrounding the tumor cells (T), and underlying the skin (S). Scale bar = 500 μm . (d) Muscle (M) tissue sample showing lower CD-31 staining than the tumor or subcutaneous tissue surrounding the tumor. Scale bar = 500 μm .

Figure 4.

(a) Bright field image of a mouse with bilateral 4T1-Luc orthotopic tumors. (b) Seed locations that were selected for perfusion analysis. (c) Pixels that fit the tumor basis function colored cyan. (d) Masks were outlined manually in ImageJ to correspond with the tumor locations visible in (b). (e) Overlay of mask region and method-generated tumor regions. Overlay was used for the sensitivity and specificity calculations.

Figure 5. Sensitivity and specificity of the method

(a) Representative tumor specific mask used to calculate sensitivity and specificity. (b) Sensitivity and (c) specificity for a single cypate run and three LS288 runs (1), (2), (3). (d) Sensitivity and specificity from b & c plotted for each run. (e) Sensitivity and specificity vs. analysis interval time post injection for LS288 run 3. (f) Receiver operating characteristic curve for different analysis intervals post injection for LS288 run 3. (g) Tumor and skin fluorescence vs. time for LS288 run 3. Injection at $t = 9$ sec (red arrow). Group A = 0 – 13 sec post injection, Group B = 13 – 24 sec and 90 – 120 sec post injection, and Group C = 24 – 90 sec post injection.