Methods for detecting host genetic modifiers of tumor vascular function using dynamic near-infrared fluorescence imaging

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Abstract: Vascular supply is a critical component of the tumor microenvironment (TME) and is essential for tumor growth and metastasis, yet the endogenous genetic modifiers that impact vascular function in the TME are largely unknown. To identify the host TME modifiers of tumor vascular function, we combined a novel genetic mapping strategy [Consomic Xenograft Model] with near-infrared (NIR) fluorescence imaging and multiparametric analysis of pharmacokinetic modeling. To detect vascular flow, an intensified cooled camera based dynamic NIR imaging system with 785 nm laser diode based excitation was used to image the whole-body fluorescence emission of intravenously injected indocyanine green dye. Principal component analysis was used to extract the spatial segmentation information for the lungs, liver, and tumor regions-of-interest. Vascular function was then quantified by pK modeling of the imaging data, which revealed significantly altered tissue perfusion and vascular permeability that were caused by host genetic modifiers in the TME. Collectively, these data demonstrate that NIR fluorescent imaging can be used as a non-invasive means for characterizing host TME modifiers of vascular function that have been linked with tumor risk, progression, and response to therapy.

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1. Introduction

NIR optical fluorescence imaging has been extensively exploited for molecular and functional imaging in small animals models and continues to be explored for additional clinical applications of fluorescence guided lymphatic and intra-operative imaging [1, 2]. Because of the high sensitivity [3] and low tissue autofluorescence in NIR, optical imaging can characterize dynamic events/changes in tissue with suitable contrast agents, and has been demonstrated for longitudinally tracking multiple pathologies and therapy response [4–9]. While, multiple organic NIR fluorescent probes nanoparticles [10], and semiconductor quantum dots [1, 11] have been developed and deployed for NIR imaging in small animals and humans [1, 10]. Indocyanine green (ICG) and methylene blue (MB) are the only FDA approved NIR probes in the US for clinical use [12]. ICG with its short half-life, higher signal-to-background ratio, lower auto-fluorescence in emission spectrum, increased tissue penetration at 820 nm compared to 700 nm in MB, and higher quantum yield is ideal for dynamic vascular function imaging [13–15]. While sharing the nonionizing behavior and the ability to do dynamic vascular imaging with MRI, NIR imaging presents several advantages such as sensitivity (femtomolar vs millimolar contrast), low cost, ease of administration and finer temporal resolution, with the major disadvantage of low 3D resolution and loss of signal to noise ratio (SNR) with tissue depth [1, 15].

To overcome the low depth resolution of 2D NIR fluorescence imaging in mouse models, Hillman et al. [15], proposed a dynamic contrast enhanced imaging system capable of capturing the in vivo bio-distribution kinetics of ICG with sub 100 ms temporal resolution and demonstrated clear resolution of internal anatomical features in ~20g nude mice through Principal Component Analysis (PCA) of ICG fluorescence kinetic curves of each 2D pixel. At the same time, numerous researchers have exploited dynamic NIR fluorescence imaging and pharmacokinetic (pK) analysis to address questions related to molecular binding of targeted contrast agents [14, 16–20], assess vascular injury [21] and comparing different patterns of vascular dysfunction [22]. However, PCA based anatomical segmentation has not been leveraged for drawing ROIs to conduct multicomartment pK analysis. In addition, PCA based internal organ segmentation methods have been restricted to mouse models only in literature. While, ROIs on tumors can be visually drawn for subcutaneous tumors in nude mice, it is challenging to identify and segment orthotopic tumors and internal organs from epifluorescence images without PCA decomposition, especially in animal models larger than 20g nude mice.

Most of the dynamic NIR fluorescence image based pK modeling literature for assessing vascular pathologies or tumor molecular imaging has been restricted to ~20g nude mouse models, with a few exceptions in canine [17] and in rabbit [23]. Further, epifluorescence based pK analysis has been limited to simple bi-exponential pK models, which only report the differences in contrast agent uptake and washout [17, 20, 23]. Recently, Choi et al. [18], and Cai et al. [19], used three compartment model to measure vascular density, perfusion and permeability parameters. Choi et al., reported an advanced pK model to isolate the tumor perfusion and permeability rates of ICG in a mouse model of breast cancer, by leveraging an image derived metric for arterial input function, which while feasible in nude mice, is not scalable to larger animal models, as clearly identifiable major arteries are not detectable in planar epi-fluorescence images for ~200g rats. We postulate, that the usage envelope of dynamic NIR imaging can be extended to assess tumor microenvironment (TME) vascular factors in advanced genetic models based on 150-200g rats, and with adaptations in imaging apparatus and integration of ICG blood concentration with kinetic imaging data, quantitative information about the effects of host genetics on tumor perfusion and permeability can be derived. We postulate further that by combining PCA based in vivo organ segmentation and multiparametric pK modeling of NIR imaging data, the analysis of vascular function and tissue perfusion can be simultaneously conducted within multiple systems (e.g., normal organs and tumor), which will provide unique insights into the impact of host genetics on the
tumor microenvironment (TME). Ultimately, we believe that this information can be leveraged to understand various physiological aspects of the vascular system that contribute to tumor risk and progression, as well as, bio-distribution of anticancer drugs and response to therapy.

Herein, we report dynamic NIR imaging with systemically delivered ICG followed by PCA-based tissue segmentation of fluorescence image stack, and three compartment pK analysis on the rat based Consomic Xenograft Model (CXM) of breast cancer. CXM is the first strategy for mapping host TME modifiers, including those that impact the tumor vasculature. Consomic rat strains differ only by a single substituted chromosome. In CXM, human breast cancer cells are orthotopically implanted into consomic xenograft host strains, which are derived from two parental strains with different susceptibilities to breast cancer. Because the strain backgrounds differ, whereas the inoculated tumor cells are the same, any phenotypic variation is due to TME-specific modifier(s) that are localized to the substituted chromosome. The parental strain for generating CXM models was the well characterized salt-sensitive or SS rat, used extensively for hypertension studies. Multiple consomic strains have been generated by sequentially replacing SS chromosomes with the outbred wild type and tumor resistant strain of Brown Normal or BN rat referred as SS.BN# and reported for their tumorigenic potential, where # refers to the chromosome no. of the BN strain substituted into SS strain via breeding [24]. These parental SS and consomic SS.BN# strains can be genetically ablated by knocking down the IL2Rγ gene, to allow grafting and growth of human cancer cell lines. Such immunocompromised strains are labelled as SS IL2Rγ SS.BN# IL2Rγ.

Previous studies on mapping of all BN chromosome substitutions in SS indicated the role of chromosome #3 in inhibiting spontaneous tumoigenesis, murine tumor growth and metastasis [24, 25]. We recently demonstrated that human triple-negative breast cancer (TNBC) tumors grown in the SS.BN3IL2Rγ CXM strain had significantly reduced growth and metastatic progression, reduced expression of notch-pathway gene DLL4 responsible for vascular budding, along with a paradoxical increase in tumor blood vessel density, compared to the parental SS IL2Rγ strain. The reduced tumor growth in conjunction with increased blood vessel density suggested that host TME modifiers on rat chromosome 3 (RNO3) induce nonfunctional angiogenesis [24, 25] but the nature of vascular dysfunction was not quantified and assessable from blood vessel staining in tissue slices, and motivated the need to develop an in vivo vascular function phenotype assessment method for CXM models.

In this study we tested the vascular function behavior of CXM models both with a TNBC (MDA-MB-231luc+) and an estrogen receptor alpha (ERα+) positive (ZR75.1luc+) human breast cancer cell line implantation model on SSIL2Rγ SS.BN3IL2Rγ host strains, via epifluorescence NIR imaging with systemic injections of ICG through tail vein in tumor bearing rats. Dynamic NIR imaging with ICG and the mathematical modeling of tumor perfusion and permeability were able to discriminate genetic modifiers of vascular function in CXM models of both TNBC and ERα+ breast cancer. Principal Component Analysis (PCA) based decomposition of time dependant epifluorescence image stacks, enabled visualization and anatomical segmentation of tumors, liver, and lungs. Regions of interest (ROIs) drawn with PCA segmented imaged were employed for the 3-compartment pharmacokinetic modeling to quantify vascular perfusion and permeability differences in CXM strains. Independent analysis of ICG plasma clearance in CXM strains via blood sampling was incorporated in pK modeling to obtain accurate strain specific arterial input function. Modeling could identify differences in systemic vasculature in CXM models which affected liver and lungs in addition to tumors. The overall pK trends observed with NIR imaging matched the MR contrast wash-in and wash-out kinetics determined with gold standard multi-slice DCE-MRI techniques. This is first demonstration of rapid and inexpensive optical imaging for quantitatively assessing the TME vascular differences driven by host genetic modifiers.
2. Materials and methods

2.1 Animal model

Immunocompromised CXM rat models SSIL2Rγ and SS.BN3IL2Rγ were generated by previously published techniques [24, 25]. Six to eight week old females weighing 200 ± 20g were selected for tumor implantation. 16 rats (n = 8 for each group) were implanted with TNBC cell line (6 × 10⁶ number of cells in right upper flank, MDA-MB-231), and 10 rats (n = 5 for each group) were implanted with ERα+ cell line (8 × 10⁶ number of cells in right upper flank, ZR75.1) tumor model. NIR imaging was performed at 10 days post-implantation for the fast-growing MDA-MB-231 model or 8 weeks post-implantation for the slower growing ZR75.1. At these time points, tumor volumes did not yet differ significantly between the SSIL2Rγ and SS.BN3IL2Rγ strains. Out of n = 8 animals implanted with MDA-MB-231 tumors, (n = 3) animals underwent dual optical (ICG) and DCE-MRI imaging on the same day to maintain consistency of tumor size and animal condition. Dual optical-MRI imaging was performed to replicate prior reported data on DCE-MRI imaging of consomic strains [25] and confirm optical imaging reported tumor vascular profile with gold standard DCE-MRI techniques for assessing tumor vasculature. Animals were anesthetized during imaging with 2.5% of isoflurane maintenance in oxygen flow of ~0.8 mL/min. Temperature was monitored and maintained at 37 °C throughout the experiment with a heating pad. For NIR dynamic fluorescence imaging, rats were injected intravenously with ICG (MP Biomedicals) and the concentration of dye was adjusted to the weight of the animal (constant ICG dose/Rat = 1.5 mg kg⁻¹), while keeping the injected volume constant. Typically, 1 mL of 400 μM ICG was injected in 200 g rat. All animal protocols were reviewed and approved by the Medical College of Wisconsin, Institutional Animal Care and Use Committee, where these experiments were performed. All rats were recovered fully after fluorescence and MRI imaging.

2.2 Imaging setup

The schematic diagram of NIR dynamic fluorescence imaging setup is depicted in Fig. 1(a). A bifurcated optical fiber bundle was used to deliver 785 nm excitation light (0.6 mW power at surface, diode laser, Thorlab Inc.,) from two positions to uniformly illuminate the entire rat body surface. A 16-bit deep cooled intensified charge-coupled device camera (PI-MAX, Princeton Instruments) was used to image the rats through computer-controlled WinView/32 software. For fluorescence imaging, a combination of 785 nm holographic notch filter [26] (SuperNotch-Plus™, HSPF-785.0-2.0, Kaiser Optical System, Ann Arbor, MI) and 830 ± 10 nm bandpass filters (830FS10-25, Andover, Salem, NH) were used and a series of 1500 frames with the CCD array hardware binned to 256 × 256 were acquired with 50 ms exposure time per frame, and a nominal gain of 10 (on instrument scale of 0–255) in a total imaging session time of 343 s. ICG dye was delivered through the tail vein 5 s after the start of image sequence using a programmable syringe pump (flow rate = 0.2 mL/s, Harvard Apparatus PHD 2000 syringe pump, Holliston, MA) and the frames acquired before ICG injection were used for the background correction. The NIR image frames can be converted to video format and clearly identify dye uptake in tumors and circulatory system, with clearance through liver and other excretory organs. (Refer to Visualization 1 for respiratory motion corrected time course images of ICG biodistribution in whole SSIL2Rγ and SS.BN3IL2Rγ rats). Visualization 1 illustrates that after 15–20s post-injection ICG biodistribution dynamics in SS.BN3IL2Rγ strain begins to deviate from that of SS strain and allows to distinguish the two strains clearly based on differences in ICG Wash-Out regime (100–300 s).

Image processing and data analysis were performed in MATLAB (Matlab 2016b, Mathworks, Nattick MA, USA) software. Following data collection, a custom designed breathing correction method (see Appendix A) with a low pass temporal filter combined with a 1D wavelet based denoising was used to filter the high frequency jitter caused by animal...
respiratory motion from the fluorescence kinetic sequences of each pixel (Visualization 2 depicts the differences in raw time course imaging (left) vs respiratory motion corrected imaging (right)). An average of pre-ICG injection frames (acquired in the ~5 s before ICG injected) was treated as background, which incorporates contributions from CCD noise, and excitation light leakage from emission filters, and subtracted from all the frames. Breath corrected and background subtracted data was decomposed by PCA in MATLAB following the previously published methods [15, 22, 27]. In addition to optical imaging, PCA has been widely used in analyzing MRI and PET data [28, 29]. Herein, PCA on dynamic fluorescence image was used to extract the spatial patterns of the internal organs associated with statistically similar kinetic behavior. Contribution of first six principal components with time basis was illustrated in Fig. 1(b).

Fig. 1. (a) Schematic diagram of dynamic fluorescence imaging setup. (b) First six principal components with time basis. (c) Variance (energy) associated with first 10 PCs. (d) Color coded and merged principal component images used to anatomically segment tumors and other organs from a single view/projection. PC-4, 5, and 6 merged as red, green, and blue channels of a true color (RGB) image (e) Three compartment Pharmacokinetic model used to fit on ICG dynamic fluorescence data and extract vascular density, perfusion $P$, and permeability parameters $K_{core}$, $K_{per}$, etc. (f) Experimentally determined blood clearance profiles, and first order kinetic model fit of ICG concentration in the plasma isolated from the SSIL2Rγ and SS.BN3IL2Rγ rat strains at different time points following tail vein injections, this time constant was used to solve the model depicted in (e). (*p ≤ 0.05; Two-way ANOVA, Error bars, s.e.m)

Energy plot indicating the variance associated with PC components is depicted in Fig. 1(c), demonstrating strong loss of energy content with successive PCs as expected. However, images until PC-6 were usable, and associated with identifiable internal organs. (Appendix B). The PC images were studied to identify best components for consistently identifying tumors, liver, and lungs, with specificity and for drawing ROIs. PC-2 to PC-4 consistently identified tumors and liver, and lungs were identified in PC-5 and PC-6. The PC components were RGB color coded and merged to visualize pixels with correlated ICG fluorescence kinetics. Fig. 1(d) depicts the Red-Green-Blue (RGB) merged image from PCs 4-5-6 for better visualization and segmentation of tumors, liver, and lungs. PCA extracted spatial patterns of the internal organs and tumor were selected to generate a mask with the help of MATLAB image processing toolbox function ‘roipoly. RGB color merging for the three PCs was performed by normalizing their peak values to 255 and then combining them into an unsigned 8-bit integer true-color image in MATLAB. The mask generated from merged PC’s (Fig. 1(d)) was used to extract the average ROIs intensity from a series of 1500 frames.
2.3 Multiparametric Pharmacokinetic (pK) model

Multiparametric pK model (Fig. 1(e)) was applied to fluorescence intensity profile in both uptake and washout phase of ICG biodistribution from PCA segmented ROIs to study the vascular heterogeneity, vascular density, permeability and perfusion rate. ICG transport between arterial compartment \( C_{\text{Vessel}} \) and the compartment of capillaries and veins \( C_{\text{Capillary}} \) is driven by perfusion rate (s\(^{-1}\)) \( P \) and can be expressed using Fick’s law [Eq. (1)]. Vascular permeability governs the bidirectional flow of ICG between intravascular and extravascular compartment \( C_{\text{Organs}} \), and can be expressed with two parameters as extravasation \( K_{\text{extra}} \) and intravasation \( K_{\text{intra}} \) rates (s\(^{-1}\)) [Eq. (2)].

\[
C_{\text{Capillary}}' = P(C_{\text{Vessel}} - C_{\text{Capillary}}) \cdot K_{\text{extra}} C_{\text{Capillary}} + K_{\text{intra}} C_{\text{Organs}} \quad (1)
\]

\[
C_{\text{Organs}} = K_{\text{extra}} C_{\text{Capillary}} - K_{\text{intra}} C_{\text{Organs}} \quad (2)
\]

\[
I = V_{\text{Vessel}} C_{\text{Vessel}} + V_{\text{Capillary}} C_{\text{Capillary}} + V_{\text{Organs}} C_{\text{Organs}} \quad (3)
\]

The overall ICG fluorescence intensity dynamics can be treated as the sum of contributions from these three compartments and can be expressed as Eq. (3). Where \( P \), \( K_{\text{extra}} \) and \( K_{\text{intra}} \) are defined in s\(^{-1}\) and \( V_{\text{Vessel}} \), \( V_{\text{Capillary}} \) and \( V_{\text{Organs}} \) are volume fraction for arterial compartment, capillary-veins compartment and extravascular-intravascular compartment respectively. Model was tested on experimentally obtained ICG fluorescence data for goodness of fit in tumors and other organs and it explained the observed ICG fluorescence kinetics sufficiently (Appendix C).

2.4 Arterial ICG concentration and lifetime measurement

In mouse work, Choi et al. [18], estimated blood clearance lifetime for ICG of 3–5 min by selecting fluorescence intensity from a pixel co-located with an artery and fitting a single exponential decay curve. While, identification of an arterial pixel might be feasible in a 20g nude mouse, it is impossible to do so consistently in 200g rats with much higher tissue scattering. Further, the blood clearance of ICG via liver and other excretion pathways can differ in SS\(^{IL2R}\)\(^{\gamma}\) and SS.BN3\(^{IL2R}\)\(^{\gamma}\) rats due to host genetics as well, and it needs to be controlled for if NIR dynamic images have to be reliably used for distinguishing TME vascular differences. The PK model fit was found to be highly sensitive to blood clearance time constant for ICG. Thus, the blood clearance of ICG was quantitatively determined by sampling blood concentrations of ICG at 5 time-points following tail vein injections. The arterial function \( C_{\text{Vessel}} \) was calculated from absorption spectroscopy on blood samples collected from tail vein over 15 min (see Appendix D). ICG concentration in blood exponentially decays as shown in Fig. 1(f) and it can be expressed by Eq. (4)

\[
C_{\text{Vessel}} = C_0 e^{-t/\tau} \quad (4)
\]

where \( t \) is time in minutes and \( C_0 \) is initial concentration of ICG. Lifetime (\( \tau \)) for SS\(^{IL2R}\)\(^{\gamma}\) (\( n = 4 \)) and SS.BN3\(^{IL2R}\)\(^{\gamma}\) (\( n = 4 \)) was determined to be 3.86 min and 2.55 min respectively. The difference was statistically significant (\( p = 0.032 \)) and thus it is critical to incorporate this information in modeling.

2.5 DCE-MRI imaging

Dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) study was performed on SS\(^{IL2R}\)\(^{\gamma}\) (\( n = 3 \)) and SS.BN3\(^{IL2R}\)\(^{\gamma}\) (\( n = 3 \)) rats on 9.4T Bruker AVANCE Scanner fitted with a volume coil to compare and contrast the results with NIR imaging. Anesthetized rats were fitted in a plastic nonmagnetic cage so that same rat can be simultaneously moved to NIR
fluorescence imaging without disturbing the animal position. A dynamically acquired T1-weighted spin-echo imaging sequence was acquired during the rapid injection of a gadolinium (0.1 mmol/kg Omniscan, Nycomed Amersham) contrast agent for a total of ~3 min. Acquisition parameters included a TE/TR = 3.78/43.93 ms, matrix = 64 × 96, slice thickness = 2 mm, and phase repetition time of 2.81 s. Three coronal slices were chosen based on the rapid acquisition rapid echo images and the tumor inoculation site. Tumor region were drawn manually on the T1-weighted images and compared with NIR imaging based segmented tumor ROI.

3. Results and discussion

3.1 NIR imaging and feature extraction

The averaged ICG fluorescence intensity for mask-segmented ROIs (lung, liver and tumor) in SS IL2Rγ and SS.BN3 IL2Rγ rats bearing TNBC tumors (MDA-MB-231) demonstrated that ICG uptake is quicker in all SS.BN3 IL2Rγ tissues and is retained longer compared to the SS IL2Rγ hosts (Fig. 2(a)-2(c)), indicating systemic differences in vascular function. Likewise, the vascular kinetics of the ERα+ tumors (ZR75.1) were similarly altered in SS.BN3 IL2Rγ in compared with SS IL2Rγ rats (Fig. 2(d)). Combined with our previous histological data demonstrating that the SS.BN3 IL2Rγ tumors have increased vascular density and tortuosity [24, 25], these data suggest that the host genetic microenvironment of the SS.BN3 IL2Rγ rat induces nonproductive angiogenesis that is reflected by the slower ICG washout. Moreover, these data substantiate the impact of TME vascular variation that is independent of tumor cell origin or molecular subtype.

3.2 Pharmacokinetic parameters

To expand upon the physiological difference(s) underlying the altered vascular kinetics in SS.BN3 IL2Rγ tumors (Fig. 2), we used a multiparametric pK approach. Regression analysis of tumor ROI at a pixel level was used to generate multiparametric maps for volume fraction and perfusion-permeability rates. PCA helps in better visualization of tumor area and allows to select ROIs with MATLAB image processing toolbox function ‘roipoly. The pK parameters obtained from tumor region (MDA-MB-231) are shown in Fig. 3(a)-3(f) where the top row represents SS IL2Rγ tumors and the bottom row represents SS.BN3 IL2Rγ tumors. Higher perfusion and lower permeability (extravasation–intravasation) rate clearly replicate the ICG kinetics trend seen in the average fluorescence intensity dynamics of SS.BN3 IL2Rγ tumors. The vascular heterogeneity and perfusion-permeability rate in CXM were quantified by pK model fitting on average intensity from the tumor ROI of SS IL2Rγ and SS.BN3 IL2Rγ strains of rats (Fig. 3(g); step wise pK model fit is illustrated in Appendix C). The higher rate of perfusion 0.31 ± 0.02 s⁻¹ in SS IL2Rγ compared to 0.24 ± 0.03 s⁻¹ in SS.BN3 IL2Rγ confirmed the increased vascular dysfunction in SS IL2Rγ and provided support for prior observations of accelerated tumor growth in SS strains [24, 25]. The pK model was also applied to other organs (Fig. 3(h)-3(i)) and the slow growing ZR75.1 (ERα+) tumor model (Fig. 3(j)), all of
which showed similarly altered vascular perfusion and permeability differences in consomic strains. The tumors implanted in SS.BN3IL2Rγ strain exhibited consistent increase in extravasation and intravasation rates, especially in the TNBC model (Fig. 3(g)), with a pronounced increase on the tumor periphery, indicating reduced blood supply to tumor interior. These results explained the progressive tumor growth retardation beginning 10 days post implantation in SSIL2Rγ strain previously observed and reported [23], but not understood at a functional level. Collectively, these data demonstrate that the SS.BN3IL2Rγ rat has systemic alterations in vascular perfusion and permeability, which support and validate histological findings and altered tumor growth and hematogenous metastasis [24, 25].

3.3 MR vs NIR imaging

The primary shortcoming of reflectance mode dynamic NIR imaging is the surface weighted nature of 2D fluorescence images, which might erroneously report on the systemic vascular changes. To confirm the systemic nature of ICG washin and washout profiles observed in (Fig. 2(a)-2(c)), a subset of tumor-bearing rats was scanned simultaneously with DCE-MRI and NIR (n = 3 per group). Representative PCA segmented NIR and averaged intensity DCE-MRI images are illustrated in Fig. 4(a)-4(b). The detailed comparison of the spatiotemporal signal enhancement and attenuation in the two strains was performed. PCA helps in generating ROI mask for tumor and liver whereas dynamic contrast MRI scan slices were imaged through tumor and liver to get corresponding area. ROI, shown in red dash, are drawn by free hand with help of MATLAB inbuilt function ‘roiploy’. The average intensity over region of tumor and liver from DCE-MRI images depicts strain differentiating characteristics as shown in Fig. 4(c)-4(d). Similar to NIR fluorescent imaging, the tumor and liver compartments of SS.BN3IL2Rγ hosts had slower washout compared to in SSIL2Rγ hosts, as detected by DCE-MRI. Notably, these differences were observed even though the MRI contrast Gd-DTPA has a substantially different molecular weight than ICG, confirming the
host dependent vascular differences observed by NIR imaging but in a 3D cross-sectional imaging setup. The advantages of 3D spatial resolution of MRI were substantially tempered by lower temporal resolution. With PCA decomposition and high temporal resolution, dynamic NIR imaging provided similar anatomical and vascular phenotypic information at a reduced expense and complexity of experiments.

Fig. 4. MRI and NIR images (PCs 2-3) acquired from (a) SS IL2Rγ and (b) SS.BN3 IL2Rγ rats showing intensity distribution from the (c) tumor and (d) liver area acquired in MRI imaging. The washout rate is higher in SS IL2Rγ compared to SS.BN3 IL2Rγ. (n = 3 in each group, *p ≤ 0.05, ****p < 0.0001; Two-way ANOVA; Error bars, s.e.m)

4. Conclusion

Multiple aspects of the TME impact cancer, yet the heritable factors that alter the TME are largely unknown, including those that modify tumor vascular formation and function. High throughput and nonionizing imaging of TME can enable rapid quantitative assessment of genetically driven vascular perfusion and permeability, thus allowing identification of TME targets of interest for oncology drug development and provide novel tools for live animal validation of mechanistic studies focused on understanding the role of TME in tumor growth, progression, and metastasis. While DCE-MRI techniques can quantify contrast agent kinetics in 3D and at clinical spatial scales, problems with temporal resolution, sensitivity, and cost of image acquisition and processing limit its utility in a high throughput preclinical imaging setting. The time resolution of small animal DCE-MRI imaging is limited to ~5 seconds. On the other hand, we have demonstrated a time resolution of 50 ms with whole body epifluorescence imaging with ICG in ~200g rats. The spatial and depth resolution limits of NIR fluorescence imaging were overcome by employing PCA segmentation of epifluorescence kinetic imaging, which allowed the visualization and segmentation of orthotopically implanted mammary tumors, and major internal organs such as liver and lungs. 3-compartment pK analysis augmented by measurements of plasma clearance of ICG in CXM strains and applied on ROIs identified by PCA segmentation, produced clear quantitative maps of TME perfusion and permeability which could distinguish the vascular microenvironment in CXM strains with identical tumors. Further, these perfusion/permeability differences in CXM strains, were independent of tumor molecular
subtype. Both the triple negative and ER + tumors identified similar differences in vascular perfusion and permeability in SS and SS.BN3 hosts, thus underscoring the role of genes on chromosome#3 in determining angiogenesis [24, 25].

In addition to providing novel mechanistic insight to the altered vascular physiology driven by host TME modifier(s) on RNO3, these data challenge the paradigm that vascular density alone is predictive of outcome. As SS.BN3 tumors have been previously reported to have higher vascular density [25], and in our results (Fig. 3(g)-3(j)) the capillary volume was significantly higher for both triple negative and ER + tumors in SS.BN3 hosts. However, counterintuitively, tumors in SS.BN3 hosts have slower growth and reduced metastasis [24, 25]. Thus, rather than only considering vascular density, altered vascular function due to nonproductive angiogenesis should be considered, in order to improve the accuracy of patient prognosis and potentially provide novel therapeutic targets for treating breast cancer. Our results indicate this nonproductive angiogenesis by reduced perfusion and increased permeability in tumors grafted in SS.BN3 hosts. (Fig. 3(g)-3(j)). These results also support pursuing the notch pathway gene DLL4, which is expressed on rat chromosome#3, and is responsible for vascular budding and growth. Prior studies reported DLL4 as highly expressed in SS hosts and suppressed in SS.BN3 hosts [25]. Lower expression of DLL4 in SS.BN3 rats might be driving non-functional blood vessel growth in SS.BN3 hosts. Current and future studies in congenic rat strains, which further subdivide chromosome#3 into regions with and without DLL4 gene, might provide specific answers to this question, with implications for developing DLL4 directed cancer therapies.

The major technical limitation of this study was the high scattering in ICG emission (~820 nm) spectrum, which limits the spatial resolution in ~200g rats. With the availability for shortwave-IR (900-1800 nm) imaging sensors, and nanoparticle and organic dyes emitting in shortwave-IR, the quality of PCA segmentation in terms of depth resolution of internal organs the spatial resolution of vasculature in tumors can be enhanced as tissue scattering is further minimized at specific windows in shortwave-IR spectrum [30, 31]. Our future efforts are directed to exploit the shortwave-IR contrast agents and imaging systems for improving TME imaging and characterization in preclinical models.

In summary, we have demonstrated that for orthotopic breast tumors, and major visceral organs such as liver and lungs, dynamic NIR imaging coupled with PCA segmentation and pK analysis provides a sufficient and effective alternative to DCE-MRI for characterizing germline dependent vascular phenotypes in subjects as large as 200 + g rats, thus opening up new avenues for preclinical cancer and vascular disease research beyond nude mouse models.

Appendix

A. Respiratory motion correction

To remove high frequency jitter caused by animal respiratory motion from the fluorescence kinetic sequences of each pixel, a custom designed correction method with a low pass filter combined with a 1D wavelet based denoising was used. A stepwise method was described as follows.

a. Filter design: Filter built using MATLAB digital signal processing tool (sptool). Parameters in filter design: LSIp (Fs = 48000 Hz, Fpass = 96 Hz, Fstop = 4800 Hz, Design Method = FIR – Equiripple, Filter order = 40, Density = 20, Wpass = Wstop = 1).

b. Padded data to the nearest $2^n$ power with function ‘wextend’.

c. Applied designed filter to padded data with function ‘filtfilt’.

d. Performed wavelet denoising on filtered data with ‘haar’ wavelet at level 5.

e. Removed padded data to go back the original dimension.
**B. Principal components contribution**

First three components contribute large amount of total energy. However, in PCA decomposition following respiratory motion correction, and spatial median filtering, we obtained usable images up to principal component (PC) 10, as demonstrated in Fig. 5 and in some cases up to PC 20. While, there are ways to draw tumor ROIs without PCA decomposition on white light or true-color images of rats, with manual judgement of tumors, it results in ambiguity for orthotopically implanted tumors in rat mammary fat pads, which often have flat and irregular topologies and not obviously visible. PCA analysis exploits the distinct vascular behavior of tumors to efficiently segment the tumor ROI from planar fluorescence kinetic images. It is almost impossible to draw liver and lung ROIs on whole body rat images, without PCA decomposition.

**C. Pharmacokinetic (pK) model fit**

Steps involved in pK model fitting on respiratory motion corrected data are as follows.

a. Respiratory motion corrected (256 pixel × 256 pixel × 1500 frame)

b. Extract organ/tumor 1D data (PCA segmented) by averaging intensity over ROI (1 × 1500 frame).

c. Normalized individual 1D data to its maximum (range 0 to 1).

d. Background subtraction by using pre-injection frames.

e. pK model fitting functions:

i. Equations (1), (2) and (3) solved using MALAB inbuilt function ‘ode45’ where the solution of the equation depends on 6 unknown parameters.

ii. Estimated unknown pK parameters from experimental data (Intensity vs time (min) from the model solution (i) with MATLAB inbuilt function ‘fmincon’ (Algorithm: ‘interior-point’, 'HessianApproximation', 'bfgs' ; Optimizations: Function Evaluations = 5000, Iterations = 1000). (model fit illustrated in Fig. 6)

iii. The lifetime, τ in Eq. (4) was 3.86 min and 2.55 min for SSIL2Rγ and SSBN3IL2Rγ respectively as determined from the ICG blood clearance measurements.
D. ICG blood clearance measurements

Arterial ICG concentration exhibits first order exponential decay with time. pK model was adapted to include this decay time constant and it was calculated by measuring the concentration of ICG in plasma at various time points from both consomics, SS IL2Rγ (n=2) and SS.BN3IL2Rγ (n=4), after single bolus injection of ICG (1.5 mg/kg). Experimental details are as follows.

Blood was collected from all the animals in heparinized syringes before the injection of ICG and blank plasma was separated and stored at –20 °C. This served as control. ICG was injected as a bolus using a programmable syringe pump (flow rate = 0.2 mL/s, Harvard Apparatus syringe pump (PHD 2000), Holliston, MA) into a catheterized tail vein of the rats. To prevent contamination of the blood samples with dye, catheter was flushed with saline two times after injection and syringe was changed. Immediately after flushing for 2 times, the blood (each 0.2 mL) was withdrawn into heparinized syringes from the catheterized tails of the rats at intervals of 1, 3, 5, 10, and 15 min after ICG injection. The collected plasma was stored at –20 °C.

The absorption spectra of the standard ICG prepared in distilled water at different concentrations was read at 450–850 nm in a microplate reader (infinite M200 PRO, Tecan, USA). The maximum absorbance was observed at 690 nm and 790 nm (Fig. 7(a)). The absorption spectra of plasma (diluted with blank plasma, 5x) collected at different time points was also read at 450–850 nm (Fig. 7(b)). The area under the curve (AUC) from standard ICG absorbance spectra plotted with concentration was used to obtain the slope by linear fit (Fig. 6).
7(c)). The concentration of ICG in the plasma at each time point was calculated from this slope. The obtained concentration of ICG at each time point was fit to first-order exponential decay to obtain a lifetime of ICG in consomic xenograft model (Fig. 7(d)). The averaged lifetime, $\tau$, measured 3.86 min and 2.55 min for SSIL2R$^{\gamma}$ and SS.BN3IL2R$^{\gamma}$ respectively.

Fig. 7. (a) The absorption spectra of the standard ICG prepared in distilled water at concentrations; 140, 120, 100, 80, 60, 40, 20, 15, 5, 1, 0.5, and 0 µg/mL. (b) The absorption spectra of plasma (diluted with blank plasma, 5x) collected at different time points. (c) The area under the curve (AUC) from standard ICG absorbance fitted with linear equation. (d) The first-order exponential decay of ICG concentration in the plasma at each time point.

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Disclosures
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