Nodal lymph flow quantified with afferent vessel input function allows differentiation between normal and cancer-bearing nodes

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Abstract: Morbidity and complexity involved in lymph node staging via surgical resection and biopsy could ideally be improved using node assay techniques that are non-invasive. While visible blue dyes are often used to locate the sentinel lymph nodes from draining lymphatic vessels near a tumor, they do not provide an in situ metric to evaluate presence of cancer. In this study, the transport kinetics of methylene blue were analyzed to determine the potential for better in situ information about metastatic involvement in the nodes. A rat model with cancer cells in the axillary lymph nodes was used, with methylene blue injection to image the fluorescence kinetics. The lymphatic flow from injection sites to nodes was imaged and the relative kinetics from feeding lymphatic ducts relative to lymph nodes was quantified. Large variability existed in raw fluorescence and transport patterns within each cohort resulting in no systematic difference between average nodal uptake in normal, sham control and cancer-bearing nodes. However, when the signal from the afferent lymph vessel fluorescence was used to normalize the signal of the lymph nodes, the high signal heterogeneity was reduced. Using a model, the lymph flow through the nodes \( L_{NF} \) was estimated to be 1.49 ± 0.64 ml/g/min in normal nodes, 1.53 ± 0.45 ml/g/min in sham control nodes, and reduced to 0.50 ± 0.24 ml/g/min in cancer-cell injected nodes. This summarizes the significant difference \((p = 0.0002)\) between cancer-free and cancer-bearing nodes in normalized flow. This process of normalized flow imaging could be used as an in situ tool to detect metastatic involvement in nodes.

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Surgical resection followed by pathological testing or biopsy of lymph nodes is commonly used to stage metastatic spread of cancer, most notably in breast cancer, melanoma, and head and neck cancer [1]. Lymphoscintigraphy after administration of radio-colloids and visual localization following injection of blue dye are the two methods typically used for lymph node mapping and identification of nodes to be resected [2]. Although this is the current standard of care, neither of these methods conveys information about the recurrence risk of specific nodes. For this reason, lymph node dissections and biopsies can be associated with over-treatment, morbidity, and complexities such as lymphedema, potential nerve damage [3], and decreased mobility, resulting in an impact on patient quality of life [4]. A mapping process that provides information about cancer presence in nodes is more ideal; this paper examines one approach to combining the clinically used paradigm with quantitative modeling and analysis of measured signals.

Studies using fluorescence mapping of lymph nodes [5,6] have focused more on localization of nodes, than estimation of tumor-burden on identified nodes [7]. The approach of using fluorescence rather than absorption or reflectance allows use of trace amounts of contrast agent (fluorophore) with real-time capability for imaging. Lymphoscintigraphy and blue dye imaging have problems such as inability to have real-time visualization; there are also practical considerations of radiotracer half-life and dye staining of the injection site, respectively. In comparison, fluorescence imaging has been shown to be feasible at micro-doses of fluorophore in human patients [8] and has a high degree of accuracy in locating sentinel lymph nodes [9].

While several studies have shown fluorescence mapping of sentinel lymph nodes in breast and skin [5,9,10] only a handful of studies have tried using fluorescence to quantitatively study lymphatic function [11]. For example, Rasmussen et al. [12] reviewed research using NIR fluorescence for lymphatic mapping and functional imaging, and a small number of attempts have been made to study fluorescence imaging to quantitatively or qualitatively identify tumor-burden on lymph nodes [13–17]. While there exists a body of research on using antibodies labeled with radio-tracers and (or) fluorophores [13], translation to the clinic has been hindered by requirement for approval of such antibody conjugates and potential concerns about insufficient specificity. Tichauer et al. [17] showed that number of tumor cells in lymph nodes correlated with epidermal growth factor receptor concentration imaged using a dual-tracer combination of a targeted and an untargeted fluorescent tracers; but that a single targeted tracer imaging would not be a reliable metric of nodal involvement [17]. While this method is attractive, it does require production of two dyes for eventual clinical use, so will have limited immediate impact.

In this paper, low dose methylene blue (MB), a dye clinically approved for use in sentinel lymph node procedures, was used to locate tumor-draining nodes with fluorescence imaging [18], to quantify lymph flow through the nodes, and test the hypothesis that kinetic parameters could be used to discriminate normal from cancer-bearing nodes. MB has been
shown to have a reasonable near-infrared (NIR) fluorescence quantum yield of ~4.4% with peak absorbance at 668 nm, extinction coefficient of 69,100 mol L$^{-1}$ cm$^{-1}$ and peak emission at 688 nm [19,20] at low concentrations. This provides the opportunity to image methylene blue at very low doses, much lower than those used in the clinic, thus avoiding problems of dye toxicity and skin staining. Methylene blue spontaneously binds with albumin through electrostatic interaction [21], and thus moves rapidly through the lymphatic system allowing quick imaging.

It has previously been shown that lymphatic vessels that supply the axillary and brachial lymph nodes drain the cutaneous regions of the forepaw [22] thus providing a simple, consistent route for fluorophore administration. The heterogeneity known to be present in fluorophore delivery to draining lymph nodes was studied together with a model of lymphatic flow, analogous to well-studied methods of deconvolution-based cerebral blood flow in dynamic contrast-enhanced computed tomography [23,24]. While there is evidence that supports lymphatic remodeling and lymphangiogenesis associated with metastasis [25], we do not attempt to study the changes in uptake patterns with progression in metastasis, but instead offer a method to identify cancer-bearing from normal lymph nodes that can be used as a preclinical test model and translated to the clinic with relative ease. To the best of our knowledge, this is the first paper to attempt to quantitatively correct non-cancer specific lymphatic uptake differences in lymph nodes using fluorescence imaging of afferent lymph vessels.

2. Materials and methods

The overall plan of this study was that an intra-nodal cancer-cell injection model was used to implant lymph nodes of rats with cancer. Then, low concentration of methylene blue was injected into the lymphatic system via footpad tissue, and imaged in vivo on a planar fluorescence scanner. Its uptake through lymph vessels and lymph nodes was measured and analyzed to compare cancer-bearing, healthy and control lymph nodes.

2.1 Cancer cell model and methylene blue preparation

Bioluminescent human breast cancer cells from MDA-MB-231-luc-D3H2LN (PerkinElmer, Waltham, MA) were cultured at 37°C in high-glucose Dulbecco’s Modified Eagle Medium (HyClone® SH30243.01, Fisher Scientific, Pittsburg, PA) supplemented with 10% fetal bovine serum (HyClone® SH30910.03, Fisher Scientific), and penicillin-streptomycin (#30-002-CI, Cellgro, Mediatech Inc., Manassas, VA). In order to ensure that all the methylene blue administered exists as MB:Albumin complex – so as to minimize the effect of two differently sized populations having different rates of uptake – MB was bound to bovine serum albumin (BSA) before use for injection.

2.2 Animal lymph node implantation procedures

All animal experiments were carried out in accordance with the Institutional Animal Care and Use Committee at Dartmouth College under approved protocols. A total of 16, 8 – 10 week old athymic nude female rats (Charles River, Wilmington, MA) were used in this study. Animals were grouped into 3 cohorts – normal (6), control (4) and cancer-bearing (6). Rats in the normal group did not undergo any surgery prior to imaging; animals in the cancer-bearing group received cancer cell injections by the procedure described below; and animals in the control group received sham phosphate-buffered saline (PBS) injections in place of cancer-cell injections.

All animals were maintained on a fluorescence-free diet for a week prior to imaging. Animals were prepped by shaving fur, and by application of Nair hair removal cream over shoulders, forelimbs, and armpits one day prior to imaging; this was done to minimize autofluorescence from fur. In order to deliver cancer-cells to the lymph node that could be visualized by fluorescence imaging in the desired imaging orientation, the animals were
imaged in a preparatory procedure (as described below) using fluorescence visible in an axillary (or brachial) lymph node to guide implantation [22]. This fluorescent node was exposed surgically while causing minimal damage to the surrounding tissue. 1 Million cancer cells in a Matrigel medium (#356230, BD Biosciences) in a total volume of 20µL were injected into this node (and any nodes visible in its vicinity) using a 27-gauge syringe needle. The area was then sutured, and the surgical site was allowed to heal for 5 days, after which fluorescence imaging was carried out to study dye uptake (as described in the next section). Control animals received 20µL of PBS instead of cancer-cells.

The cancer bioluminescence was imaged to confirm cancer presence in nodes, using Luciferin (#88294 Thermo Scientific) administration, prior to fluorescence imaging using the Xenogen VivoVision IVIS bioluminescence system (Perkin Elmer, Waltham, MA). A total of 6 animals were found to exhibit tumor presence in at least one lymph node at 5 days post tumor injection. Cancer cells remained confined to the inoculated nodes and no spreading to nearby nodes was seen.

2.3 In vivo fluorescence lymph imaging

Prior to imaging, each animal was anaesthetized using 1.5 - 3% isoflurane (Piramal, Bethlehem, PA) in 1.5 L/min oxygen. It was then placed on its side with the forelimb on one side of the body stretched out so as to expose lymph nodes of the axilla; shown in Fig. 1. Next, 25 nmoles of Methylene Blue (Sigma-Aldrich, St. Louis, MO), pre-bound to bovine serum albumin (Sigma-Aldrich, St. Louis, MO) in a 20 µl volume of PBS, were injected intra-dermally using a 27-gauge syringe-needle into the base of a forepaw footpad of each animal. Care had to be taken to ensure the injection was not deeper than ~0.5 mm; deep injections lead to delayed uptake of dye into the lymph system, as it is the dermis that is richly supplied with lymph capillaries. 3 out of 16 animals required their paws to be massaged for 30 seconds, to ensure fluorophore uptake by lymph capillaries. It has previously been shown that lymphatic vessels supplying the axillary and brachial lymph nodes drain the
cutaneous regions of the forepaw [19,20,22]. Additionally, the large injection volume produced pressure that caused the lymphatic capillaries to take up dye.

All fluorescence imaging was performed using a PearI® Impulse system (LI-COR Biosciences, Lincoln, NE) which provides planar surface images in the near infrared band [17,21]. The system was set up to acquire grayscale white light, and 700 nm channel images at 2 frames per minute for 40-60 minutes. The 700 nm channel setting uses a 685 nm laser diode excitation source with an intensity distribution of 2 mW cm$^{-2}$ and a 720 ± 20 nm bandpass emission filter. The imager has a resolution of 85µm and a 11.2cm x 8.4cm (1300x964 pixels) FOV. The timing allowed capture of the dynamics of lymph flow through paws, lymph vessels, and lymph nodes.

2.4 Modeling based estimation of nodal lymph flow

Interpretation of the flow dynamics of lymph nodes is complicated by the large heterogeneity seen in supplying afferent lymph delivery, and the known complexity of pseudo-compartments present in the nodes, which are incompletely separated by thin trabeculae. Compartment kinetic modeling of the flow requires identification of the minimum number of pertinent regions affecting the observed flow, which can be difficult.

In this study, a nonparametric deconvolution method was developed to mathematically estimate the lymph flow across the node, based on dynamic MB fluorescence data – an approach analogous to methods used in cerebral blood flow characterization in CT perfusion [23,24] and near-infrared spectroscopy [26, 27]. To described this model, we assume $L(t)$ is the uptake of dye into lymph node, $C_a(t)$ is the fluorescence signal from the afferent lymph vessel, $R(t)$ is the impulse residue function of the lymph node in question, and is the nodal lymph flow. The following equation describes the convolution governing the tissue kinetics.

$$L(t) = F_{LN} \cdot R(t) \ast C_a(t)$$

(1)

Here, “ $\ast$ ” represents the convolution operator. $L(t)$ and $C_a(t)$ are measurable quantities and deconvolution of $C_a(t)$ from $L(t)$ produces a scaled version of $R(t)$ whose peak can be used to estimate nodal lymph flow ($F_{LN}$). $L(t)$ and $C_a(t)$ were measured over the duration of imaging by manually selecting regions-of-interest over the lymph node and afferent lymph vessel respectively, followed by data smoothing which preserved peak intensities and peak positions. Equation (1) was solved in the least squared sense to recover $F_{LN} \cdot R(t)$ . By definition, $R(t)$ is the fraction of dye remaining in the lymph node after an idealized bolus injection (Dirac-delta or “impulse” function) into the afferent vessel. To stabilize the inversion of Eq. (1), physiological constraints were applied, which included the constraint that there was no retrograde flow (and hence $R(t)$ is monotonically decreasing). Additionally, $R(t)$ reflects the case where all dye enters the node at the same instance in time, so it has a maximum fractional value of 1. This enables the recovery of $F_{LN}$ , since it is equal to the maximum of $F_{LN} \cdot R(t)$ , which is the parameter recovered by inversion of Eq. (1). Additionally, nodal lymph volume $V_{LN}$ was calculated by integrating $F_{LN} \cdot R(t)$ over time and mean transit time, $\bar{t}_{LN}$ of lymph through the node was calculated as $\frac{V_{LN}}{F_{LN}}$ [25], according to the central volume principle. More complete details regarding the use of the physiologically constrained deconvolution is found elsewhere [28].
Fig. 2. Early time-point frames (white light image overlaid with red pseudo-colored fluorescence image) for various normal, sham control and cancer-bearing rats are shown. Yellow arrows point to nodes and green arrows point to afferent lymph vessels. (a-d) Normal rats imaged, images are tagged with “N” to indicate cohort. e-g) Rats from sham control cohort, indicated by letter “S”. (i-l) Rats from the cancer-bearing nodes cohort, images are tagged with letter “C”, also asterisks indicate cancer-bearing nodes. Fluorescence from injection site was blocked off manually in the overlay. (h) shows fluorescence from an exposed lymph vessel on which segments separated by valves within the vessel can be clearly demarcated. This is the only image in this panel showing exposed lymphatics, lymph node is not visible here. Lymphangions are visible and their lengths were used to inform ROI selection in all animals.

It should be further noted that $L(t)$ and $C_a(t)$ were in units of fluorescence, but were measured on the same series of images; therefore, they are both linearly related to concentration of methylene blue by approximately the same factors (e.g., quantum efficiency, absorption, scatter, and detector efficiency) as long as the tissue optical properties at both locations are not significantly different. For this reason we present the values in the conventional units of blood flow: millilitre of lymph fluid, per gram of tissue, per minute (ml/g/min).

2.5 Image data analysis

The 22-bit images from the Pearl imager were read using Bio-Formats plug-in (LOCI, University of Wisconsin-Madison) and analyzed using a combination of ImageJ and Matlab (Mathworks, Natick, MA) software. Fluorescence measurements were made on temporal 700 nm channel images by calculating the mean signal intensity from regions-of-interest over lymph nodes, lymphatic vessels, and skin free from fur and melanin. Pre-injection measurements were subtracted from all post-injection measurements to eliminate autofluorescence. Additionally, build-up of fluorescence in the skin over the duration of imaging necessitated background subtraction, for which mean signal from the selected skin...
region was used. Choice of ROI size for lymph vessels was based on lymphangion length measured from one animal whose exposed lymph vessels clearly showed segmentation on a lymph vessel indicating lengths separated by valves. This is shown in Fig. 2(h). Measurements on normal, sham control, and cancer-bearing nodes were tested for statistically significant differences using two-tailed Welch’s t-tests, using Microsoft Excel.

3. Results

Figure 1 shows an example of fluorescence imaging of a lymph node in a rat during and post implantation. Corresponding *in vivo* and *ex vivo* bioluminescence images are shown; these confirmed cancer presence in the node(s) imaged post implantation. A comparison of Figs. 1(a)-1(b) and Figs. 1(d)-1(e) qualitatively confirms that the direct-injection tumor model successfully produces a tumor-bearing lymph node in a very short time frame of 5 days with minimal disruption of lymphatics while ensuring that this node drained the paw and could be imaged using methylene blue. Animals that went through surgeries had porphyrin-rich surgical scars near the axilla, which had to be covered with shrouds to minimize autofluorescence from them.

Figure 2 shows single frames at early time-points post methylene blue injection for various animals. Dye delivered to the paws was successfully taken up by axillary lymph nodes. However, lymphatic routes taken by the dye varied across animals. Among the normal and sham control animals, a few rats showed uptake into two lymph nodes (see Figs. 2(c), and 2(e)), while all others showed uptake into one node each. Among the cancer group, at least one rat showed dye uptake into two nodes (see Fig. 2(l)): both these nodes were visible at the time of cancer-cell implantation and both were accordingly inoculated with cancer cells. Figure 2(h) demonstrates the ability to visualize segments (lymphangions) along a lymph vessel when the vessel is exposed. Fluorescence from lymph vessels appeared to fluctuate in intensity spatially and temporally along the vessel length. Temporal variation was attributable to the pulse-like flow of lymph fluid through the lymph system, while spatial variation was attributable to differences in lymph vessel depth at various locations. Furthermore, in several animals, dye took routes through ventrally located vessels, as opposed to the dorsal lymphatic routes seen in the majority of animals. All this indicates significant delivery differences, leading to differences in uptake of dye into the corresponding node(s).

Figure 3(a), 3(b), 3(d), 3(e) demonstrate temporal plots of fluorescence intensity in nodes, and afferent lymph vessels; fluorophore injection was performed at t = 0 minutes. Various measurements were used to study variation within each cohort, and compare normal, control and cancer-bearing nodes. Average node and vessel fluorescence was calculated on measured fluorescence data. 74% (standard deviation from mean) variability in average fluorescence signal was seen among normal healthy nodes, and a variability of 59% was seen in the average fluorescence signal from their feeder vessels. Control nodes showed 60% and 118% variability in average fluorescence in nodes and afferent vessels respectively. Cancer-bearing nodes (indicated on Fig. 2(i)-2(l) with asterisks) exhibited 105% and 61% variability in average fluorescence from nodes and lymph vessels, respectively. Peak fluorescence from nodes showed slightly lower variation – of 58%, 51% and 102% in normal, control, and cancer-bearing nodes, respectively – than average node fluorescence. This likely arose from large differences in retention at late time points among all nodes (88%, 77%, and 152% respectively for normal, control, and cancer nodes), contributing to the increased variability in average fluorescence.

From Fig. 3, one can also observe that the time at which peak-MB-fluorescence appeared varied among nodes. This arrival time had 62%, 63% and 78% variation in cohorts respectively, with no significant differences between the groups. We looked at time-of-arrival of peak-fluorescence intensity, relative to injection time, and peak fluorescence signal from lymphatic vessel. In an attempt to eliminate the subjectivity of these measurements to injection administration and time delay between injection and image acquisition, time delay...
between peak fluorescence in nodes and vessels was calculated as the difference in their peak
arrival times respectively. However, owing to the large heterogeneity in flow through lymph
vessels, this quantity showed large variability (>100%) in all groups.

The peak fluorescence intensities from afferent lymph vessels (\(P_a\)) were used to normalize
the fluorescence signal in the lymph nodes downstream from them. This normalization
process reduced variations in average node fluorescence to 75%, 37% and 65% in each cohort
respectively, which appeared to be an improvement over the raw intensity analysis. Of all the
quantities tested, the measurement with lowest variability (39% in normal, 23% in control,
and 44% in cancer-cell injected nodes) was the peak-normalized node fluorescence (can also
be called ratio of peak node fluorescence to peak vessel fluorescence, \(\frac{P_N}{P_a}\)). Moreover, both
normalized node fluorescence measurements showed statistically significant differences (\(p <
0.01\)) between the cancer-bearing and cancer-free cohorts.

Table 1 reports mean ± standard deviation and intra-group variation for the various
measurements, and p-values from Welch’s two-tailed t-tests between cancer-free and cancer-
bearing cohorts. Rows 1 through 11 show parameters measured directly from measured
fluorescence signals. Box plots comparing some of the important measured parameters from
all cohorts are shown in Fig. 4.

Fig. 3. Lymph node fluorescence, afferent vessel fluorescence and impulse residue functions
are shown for all animals. Animals are color and marker-coded across columns.
Table 1. Various parameters used to compare normal (A), control (B), and cancer-injected (C) nodes. * indicates strongly significant differences ($p < 0.01$); † indicates significant differences ($p < 0.05$)

| # | Parameter | Normal Animal Cohort (A) | Sham Surgery Cohort (B) | Cancer-bearing Cohort (C) | p-value from Welch’s T-test between groups |
|---|-----------|--------------------------|-------------------------|---------------------------|------------------------------------------|
| 1 | Average Node Fluorescence ($A_\text{N}$) | 0.035 ±0.026 (74%) | 0.02 ± 0.012 (60%) | 0.028 ± 0.033 (118%) | 0.27 0.63 0.70 0.94 |
| 2 | Average Afferent Vessel Fluorescence ($A_\text{A}$) | 0.005 ±0.003 (59%) | 0.0025 ± 0.003 (118%) | 0.009 ± 0.007 (77%) | 0.30 0.08 0.18 0.12 |
| 3 | Peak Node Fluorescence ($P_\text{N}$) | 0.096 ±0.056 (58%) | 0.049 ± 0.025 (51%) | 0.088 ± 0.09 (102%) | 0.11 0.35 0.86 0.79 |
| 4 | Peak Vessel Fluorescence ($P_\text{A}$) | 0.037 ± 0.02 (54%) | 0.02 ± 0.013 (65%) | 0.07 ± 0.047 (67%) | 0.13 0.05 0.17 0.10 |
| 5 | Retained Node Fluorescence (at 40 minutes post injection) | 0.026 ±0.023 (88%) | 0.013 ± 0.01 (77%) | 0.023 ± 0.035 (152%) | 0.25 0.54 0.86 0.90 |
| 6 | Time of Peak Node Fluorescence (mins) | 2.74 ± 1.69 (62%) | 2.89 ± 1.81 (63%) | 3.39 ± 2.65 (78%) | 0.90 0.73 0.62 0.63 |
| 7 | Fluorescence peak delay vessel to node (min) | 0.98 ± 1.05 (107%) | 0 ± 3.63 (NA) | 1.26 ± 2.43 (193%) | 0.60 0.54 0.80 0.57 |
| 8 | Average Normalized Node Fluorescence ($A_\text{N}/P_\text{A}$) | 0.95 ± 0.71 (75%) | 1.1 ± 0.41 (37%) | 0.31 ± 0.2 (65%) | 0.67 0.02† 0.08 0.004* |
| 9 | Average Normalized Afferent Vessel Fluorescence ($A_\text{A}/P_\text{A}$) | 0.13 ± 0.08 (62%) | 0.1 ± 0.07 (70%) | 0.13 ± 0.05 (38%) | 0.48 0.49 0.88 0.77 |
| 10 | Peak Normalized Node Fluorescence ($P_\text{N}/P_\text{A}$) | 2.72 ± 1.07 (39%) | 2.73 ± 0.64 (23%) | 1.04 ± 0.46 (44%) | 0.97 0.006* 0.01* 0.0002* |
| 11 | Normalized Retained Node Fluorescence | 0.7 ± 0.7 (100%) | 0.63 ± 0.39 (62%) | 0.21 ± 0.24 (114%) | 0.85 0.12 0.15 0.04 |
| 12 | Nodal Lymph Flow ($F_\text{LN}$) | 1.49 ± 0.64 (43%) | 1.53 ± 0.45 (29%) | 0.5 ± 0.24 (48%) | 0.90 0.01* 0.01* 0.0002* |
| 13 | Nodal Lymph Volume ($V_\text{LN}$) | 6.18 ± 4.25 (69%) | 19.39 ± 15.48 (80%) | 2.19 ± 1.06 (48%) | 0.19 0.11 0.07 0.03† |
| 14 | Mean Transit Time ($t_\text{LN}$) [mins] | 4.18 ± 2.03 (49%) | 11.42 ± 6.59 (58%) | 4.61 ± 1.24 (27%) | 0.11 0.13 0.66 0.21 |

The large amount of inherent heterogeneity present in fluorophore uptake by healthy nodes was hypothesized to be attributable to heterogeneity in delivery. As seen above, our imaging method provided the opportunity to make measurements on lymphatic vessels thus facilitating their use in accounting for such delivery differences.
In an attempt to develop a model that would combine correction of heterogeneity in lymphatic delivery with a model that could provide estimates of a physiologically relevant quantity, as described in the methods section, an afferent vessel input model using nonparametric deconvolution with physiological constraints was used to characterize the relationship between lymph vessel fluorescence signal, \( C_v(t) \), and nodal fluorescence signal, \( L(t) \). Deconvolution of \( L(t) \) and \( C_v(t) \) resulted in the recovery of \( F_{LN} \cdot R(t) \), the impulse residue function, which contains information about lymph flow through node \( F_{LN} \), lymph volume in the node \( V_{LN} \), and mean transit time \( T_{LN} \) — the mean time between when the lymph enters from the afferent vessel and exits by the efferent vessel. Node fluorescence \( L(t) \), vessel fluorescence curves \( C_v(t) \), and the corresponding \( F_{LN} \cdot R(t) \) functions are shown in Fig. 3 for all cohorts. These curves were then used to estimate \( F_{LN} \) as described earlier. Figure 5 shows \( F_{LN} \), \( V_{LN} \), and \( T_{LN} \) plotted for all animals from normal, control and cancer-bearing cohorts. Two-tailed Welch’s t-tests revealed significant differences between \( F_{LN} \) of normal and cancer-bearing groups (\( p = 0.01 \)) and control and cancer-bearing group (\( p = 0.01 \)). Furthermore, while normal and sham control nodes show differences in fluorophore uptake in Rows 1-7 of Table 1, due to inherent heterogeneity and possible also due to inflammation from surgery, measurements based on afferent lymph vessel normalization such as in rows 8, 10, and 12 of Table 1, reveal high degree of similarity (\( p = 0.9 \)) between these groups. For example, t-test between \( F_{LN} \) of normal and control groups revealed a large p-value (\( p = 0.9 \)) indicating no significant differences between these groups, thus allowing us to combine both cancer-free groups. A t-test on this quantity between all cancer-free and cancer-bearing animals showed a p-value of 0.0002, which indicated highly significant differences. \( F_{LN} \) is closely related to the previously reported ratio of peak fluorescence in node to vessel fluorescence as shown.
they had a Pearson correlation coefficient of 0.7. $F_{LN}$ was calculated to be 1.49 ± 0.64 ml/g/min in normal nodes, 1.53 ± 0.45 ml/g/min in sham control nodes, and 0.5 ± 0.24 ml/g/min cancer-cell injected nodes. Additionally, $V_{LN}$ was 6.2 ± 4.3 ml/g, 19.4 ± 15.5 ml/g, and 2.2 ± 1.1 ml/g in normal, control, and cancerous nodes, respectively; and $\overline{I}_{LN}$ for the normal and cancerous nodes was 5.7 ± 1.7 min and 5.2 ± 0.8 min, respectively. Neither parameter showed significant differences between cancer-bearing and control nodes ($p = 0.11$ and $p = 0.13$ respectively). Rows 12–14 of Table 1 summarize these measurements along with p-values from Welch’s t-tests between various cohorts, and Fig. 5 shows boxplots compare these modeled parameters for various cohorts.

4. Discussion

This proof-of-principle paper presents experimental evidence on the ability to use fluorescence measurements of methylene blue (MB) to identify, with statistically significant difference, cancer-bearing from cancer-free lymph nodes in small animals, by using afferent vessel input functions. This is important because MB is commonly used in surgical oncology applications [20] including sentinel lymph node procedures [18], and so it is possible that its kinetics could be better exploited for further information. The small size of MB (~300 Da), combined with its affinity for albumin in lymph, makes its movement through the lymphatic system very swift and uptake to nodes is seen within the first 5 minutes of injection. This feature allows immediate imaging with little or no wait time post fluorophore administration. Additionally, short imaging-durations of 10-20 minutes could produce results similar to those presented (~40 minutes of imaging), as most of the information is contained in the peak of fluorescence signal. While this study focused on using fluorescence imaging, the presented methods have the potential to be extended to dynamic absorption or molecular imaging as well.

Implantation of bioluminescent breast cancer cells directly into the nodes worked well to directly confirm cancer-cell presence in nodes. Animals imaged for MB fluorescence prior to tumor implantation, showed that lymph nodes of the axilla drain the cutaneous and subcutaneous regions of the forepaw on their side of the body. A large amount of variability was observed in the uptake of fluorophore into a draining lymph node (~75%), this variability was attributable in a large part to inconsistency in administration, inherent differences in lymphatic vessels, differences in pressure within the lymphatic system, et cetera. Figure 2 shows uptake pathways of methylene blue in various healthy and cancer-bearing animals. Within the group of healthy animals that had never been through surgery, significant differences were seen in the lymphatic trafficking routes of the dye. In most animals from our
study, lymph was supplied from paw to node via a dorsal lymph vessel. In some cases, animals showed ventral or highly branched routes. On the other hand, a few animals showed multiple routes within the same animal, and uptake by multiple nodes as well. Furthermore, images in Fig. 3 show high variation in intensities and shapes of uptake curves in both vessels and nodes; a few vessel inputs show double peaks, which could be attributed to lymph propulsion by massaging of the animal paw. It must be noted that in our imaging set up, only those vessels that supplied the bulk of the dye to the draining node and those that were close to the skin were visible. For the purposes of this study and for the sake of simplicity, it was assumed that all input to a visible node arose from this visible vessel, which is a reasonable assumption given the superficial depth of injection of the MB in the paw. However it is possible that more branches of a lymph vessel could have fed a single node beyond what was imaged, and this could lead to more complex kinetics which may not have been modeled [17] in this study [29].

The cell-line [17,30] is known to metastasize from a primary tumor to draining lymph nodes in host mice, over a period 10-weeks, but here was examined for establishment of a short term model for lymph node involvement. An intra-nodal cancer-cell injection model has distinct advantages for research imaging studies, because the time to incubate the tumor is dramatically reduced. In this study, the imaging was done at ~5 days post surgery, versus 10 weeks for metastatic invasion from a primary tumor. In this approach, the cancer-cells were easily deposited into lymph nodes with minimal damage and the animal’s lymphatics did not appear to change significantly over a period of 5 days or as a result of surgery. No significant differences were seen between normal and cancer-bearing nodes when average nodal fluorescence was used as the basis to compare the control and cancer-bearing animals. This was attributable for the most part to significant delivery differences (>50%) through the feeder lymph vessels.

Several parameters based on temporal fluorescence measurements in lymph nodes and lymph vessels were used to differentiate control from cancer-cell bearing nodes. All methods that relied solely on the fluorescence signal from lymph nodes failed owing to the large heterogeneity in lymphatic delivery to these nodes (Table 1). On the other hand, methods based on nodal fluorescence normalized by peak fluorescence from respective vessels performed fairly well, with the ratio of peak fluorescence in node to peak fluorescence in vessel \( \frac{P_N}{P_I} \) producing the most significant difference between normal and cancer-bearing nodes. Overall, no significant differences were seen between normal surgery-free and sham-control groups, and the differences (not statistically significant) observed between them in average nodal fluorescence are eliminated when afferent vessel normalization is used.

In order to estimate a physiologically relevant quantity that correlated with presence of cancer in lymph nodes, an afferent vessel input model of lymphatic flow through nodes, analogous to the arterial input model of kinetic imaging used to measure cerebral blood flow, was used [23,26,27]. Based on this nonparametric model, a scaled version of lymph node impulse residue function was calculated by physiologically-constrained deconvolution [28] of afferent vessel input from node fluorescence to estimate lymph flow through node, \( F_{LN} ; F_{LN} \) is very closely related to \( \frac{P_N}{P_I} \), and is significantly different in cancer-bearing nodes as compared both to control and normal nodes. Furthermore, there was striking similarity between \( F_{LN} \) of normal and control nodes that received sham PBS injections, thus indicating that inflammation due to surgery did not significantly contribute to nodal lymph flow. Normal nodes showed nodal flow rates ranging from ~0.5-2.5 ml/g/min; this was comparable to the values of lymph flow rate reported elsewhere [11,31].
One of the benefits to using a nonparametric model to perform kinetic analysis is that no explicit assumptions are necessary regarding the structure or compartmentalization of the node. Therefore, the shape of the $R(t)$ reflects the true kinetics of the node, rather than an imposed model. Once an understanding of the general dynamics is gained, more specific models can be applied where suitable, for example, to quantify fast and slow components or to estimate binding if the tracer is targeted [17]. These may offer improvements in specificity and sensitivity, when applied to clinical cancer models. While this paper did not attempt to study structural changes in lymphatic vessels supplying cancer-bearing lymph nodes [32], it can be stated with confidence that any changes in the lymphatic architecture will directly affect lymph delivery via lymph vessels to a node downstream of this channel, our method will nevertheless be able to objectively correct for this delivery difference and assess cancer presence in the draining lymph node, but will not be able to detect cancer spread in the lymph vessels themselves.

While the presented methods and results provide evidence to support our claim that $F_{LS}$ can be used to identify cancer-free from cancer-bearing nodes, this approach is not proposed as an alternative to other existing methods of metastasis detection such as $^{18}$F-FDG PET imaging, but as complementary to existing fluorescence mapping and surgical resection techniques. Furthermore, based on the presented methods and results, this approach can potentially identify advanced stage nodal involvement, and we expect that reduction of nodal lymph flow rates due to metastasis, would be proportional to cancer-cell density in the nodes. However, further testing and analysis would be required to establish sensitivity and specificity limits on this system. A technological limitation of this approach that could hinder translation to a clinical environment may be the limited depth sensitivity of 700 nm fluorescence signal combined with significant tissue autofluorescence in the 700nm window. This may be partly overcome by use of 800 nm fluorophores such as ICG [5,20] or IRDye® 800 CW [33] as the tissue autofluorescence in the 800 nm window is very low and nodes within ~1-2mm depth can be imaged using NIR fluorescence intraoperatively. However to non-invasively image deeper nodes, fluorescence tomography systems with subsurface imaging and fluorescence quantification capabilities such as the ultrasound-guided fluorescence tomography system described here [34] may need to be used. Such systems are capable of imaging down to ~1cm. As future work we propose use of an adaptation of this system to image fluorescence in lymph nodes, this would have a higher translatability into the clinic to image lymph nodes of the head, neck, and possibly axilla.

5. Conclusions

This study shows large inherent heterogeneity exists in lymphatic delivery to lymph nodes of the axilla in this rat model, for both normal and cancer bearing animals. An intra-nodal cancer-injection model was employed and we demonstrated that incorporation of an afferent vessel input model could successfully account for inherent uptake variability, revealing similarity between normal and control nodes but significant differences between cancer-bearing and cancer-free (normal and sham control) nodes. Lymph flow in cancer-cell injected nodes is significantly lower than normal nodes, possibly due to cancer-cell mediated disruption of flow through nodes.

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