

# Indocyanine Green Lymphography Imaging of Normal Lymphatic Drainage in the Lower Limbs

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## Abstract

**Objectives:** Indocyanine Green Lymphography (ICGL) has emerged as a potentially powerful tool for the study of the superficial lymphatic system and to support the diagnosis of lymphoedema. However, detailed descriptions of ICGL findings in healthy individuals are limited. In this study, we imaged a series of healthy participants using ICGL, attempting to establish quantitative and qualitative ICGL parameters of the lower limb.

**Methods:** Sixteen healthy individuals aged 20-55 years were recruited to undergo lower limb ICGL after 0.1mL injections of 1g/L ICG were administered intradermally to five locations around the foot. Outcome measures included: i) the drainage routes of contractile lymphatic collectors observed, ii) the number of lymphatic vessels crossing the anterior ankle and iii) the pumping frequency of lymphatic vessels. Abnormal features, such as highly tortuous or vessels with retrograde lymph flow, were noted.

**Results:** Propulsion of ICG containing lymph could be seen in all individuals, with drainage via the anteromedial and anterolateral drainage pathways predominating (observed in 31/32 and 25/32 limbs respectively). The number of lymphatic vessels crossing the anterior ankle was  $3.4 \pm 1.1$  with an average rate of 1 propulsion every 66 seconds in the vessels investigated. Isolated cases of highly tortuous and refluxing vessels were observed.

**Conclusions:** Although limited by absorption and scatter of infrared light, ICGL facilitated the characterisation of normal lower limb lymphatic vessels through a rigorous set of objective measures. This in turn will allow better identification of pathological changes.

**Advances in Knowledge:** Establishment of normal lower limb lymphatic anatomy and function.

## Keywords

Indocyanine Green Lymphography (ICGL); Near-Infrared Fluorescence (NIRF); Lymphatic System; Healthy Controls; Lower Limb

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## Introduction

The lymphatic system has historically been challenging to image, which has limited the understanding of the lymphatics and their contribution to disease. Comprised predominantly of fragile, sub-millimetre vessels, filled with colourless fluid, lymphatic cannulation and delivery of contrast agents for radiographic lymphangiography is technically demanding with potentially severe side effects including pulmonary embolism (1). Lymphatic flow is also slower than blood circulation and erratically pulsatile, making techniques such as Doppler ultrasound not generally applicable to the study of the lymphatics (2).

Indocyanine Green (ICG) lymphography (ICGL) is an imaging technique which offers high spatial and temporal resolution visualisation of superficial lymphatics compared to the more routinely used lymphoscintigraphy (3). Relying on preferential trafficking of the ICG into lymphatic vessels following interstitial injection, ICGL does not require direct lymphatic cannulation (2). Illumination with near infrared (IR) radiation, and detection of the shifted IR emission, may also be performed with small portable devices providing immediate depiction of the lymphatics. ICGL can therefore be used in the clinic to allow real time and in vivo imaging of the superficial lymphatic vessels with no ionising radiation exposure. Results including the identification of lymphatic pathways, and whether the flow within them is functional or disrupted, can then guide and assess therapies for lymphoedema such as manual lymphatic drainage and lymphovenous anastomosis surgery (4,5).

ICGL provides valuable novel insights into the functioning of the superficial lymphatic system. Previous studies have used ICGL to study the lymphatics in the extremities of patients with primary and secondary lymphoedema and demonstrate benefits over alternative methods of investigation (6–10). To recognise pathological abnormalities, one must know what is normal, and yet few reports of lymphatic vessels within normal legs of healthy volunteers have been published. Many studies have imaged the non-oedematous limb of unilateral lymphoedema patients as a comparator to an affected limb, however these limbs could conceal incipient lymphoedema, while studies of cadavers cannot capture true lymphatic physiology (11–13).

In this study, we aim to provide normal parameters for the anatomy and physiology of the superficial lymphatic vessels in the lower limbs of healthy individuals without clinical symptoms of lymphatic disease using ICGL.

## Methods

### Participants

Healthy volunteers recruited to undergo ICGL in the lower limbs at \*\*\*\* between 21/12/2022 and 21/11/2023 had their ICGL data reviewed in this observational study. Participants included staff members recruited following an internal recruitment email, and friends or partners of existing patients, but not their blood-related relatives. Prior to enrolment participants were screened for any history of lymphatic disease and, to ensure their safety to undergo ICGL, any known allergy to ICG, sodium iodide or iodine. Pregnant individuals were also not imaged.

Ethics approval for this research was granted by \*\*\*\*, reference \*\*\*\*.

### ICG Contrast Injection Protocol

To facilitate ICG uptake into each of the four lymphatic vessel groups of the lower limbs (anteromedial, anterolateral, posterolateral and posteromedial), ICG was administered into 5 circumferential locations in each foot: 1<sup>st</sup> and 4<sup>th</sup> web spaces, lateral midfoot and lateral and medial rear-foot (Fig 1) (14,15). Each injection consisted of 0.1ml of a 1g/L mixture comprising 25mg of ICG (Verdye™) dissolved into 20 ml water for injection and 5ml 1% lidocaine. ICG was administered manually through intra-dermal injections using a 29G needle and 1 ml syringe. Following injections, participants were asked to perform foot and toe flexions ten times before immediate commencement of imaging.

### Imaging Protocol

ICGL was performed with a Fluobeam® 800 (Fluoptics, Grenoble, France), capable of exciting the area under investigation with IR radiation at 750nm and detecting the emitted IR with a single optical head. The optical head was used either freehand or mounted on an articulating arm to improve camera stability whilst imaging. A distance of 15-20cm between the optical head and the skin was generally maintained as per the manufacturer's guidance. Moving beyond this range was acceptable if the operator believed they could achieve improved focus or sensitivity by doing so. The operator also had control of the infrared exposure time and background light level emitted by the optical head, however an exposure time  $\leq 40$ ms was generally employed as this ensured the maximum achievable image frame rate of 25 frames per second. Focussing was automatically performed by the imaging system. No offline processing of recorded still or video image data was performed.

Imaging consisted of a mobile phase to document the overall pattern of lymphatic enhancement within a limb and separate videos of 3-5 minutes focusing on, for example, the anterior ankle as well as other anatomical regions where lymphatics could

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4 be identified. The optical head was mounted in the articulated arm during all video  
5 capture.  
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8 Checking the integrity of one-way lymphatic collector valves by the application of  
9 manual pressure down the limb was routinely performed, as was imaging the rear of the  
10 limb with the participant standing if they could comfortably do so.  
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## 12 13 Image Analysis

14 Employing the classification described by Shinaoka et al. (14,15) lymphatic vessels  
15 were categorised based on the path taken in the limb as belonging to one of four  
16 groups: anteromedial, posteromedial, anterolateral and posterolateral. Additional  
17 imaging measures of interest were: i) the number of lymphatic vessels crossing the  
18 anterior ankle (approximately at the level of the talus), ii) the pumping frequency within  
19 one of these ankle vessels (i.e. the number of times a vessel emptied during continuous  
20 video acquisitions of at least 3 minutes divided by the video duration) and iii) imaging  
21 signs considered of an abnormal nature.  
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24 Potentially abnormal imaging features included lymphatic vessels that were highly  
25 tortuous or directed across, or back down, the limb, the presence of lymph nodes,  
26 regions of dermal backflow and lymph stasis (i.e. not being drained from the injection  
27 site). Instances where lymph was manually drainable back down the limb was also  
28 considered abnormal.  
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31 Notes taken during the imaging examination formed the backbone of the analysis.  
32 These records, and the corresponding images/videos from the ICGL session, were then  
33 interrogated further by an imaging scientist with 6 years of experience in lymphatic  
34 imaging (\*\*\*) to ensure the accuracy of the details recorded in the notes and to  
35 supplement where details were lacking. At this review stage estimates of lymphatic  
36 pumping frequency in a single anterior ankle lymphatic of each limb were also made. In  
37 addition, in 10 healthy control ICGL videos where the average pumping rate closely  
38 matched that of the entire dataset, lymphatic pumping frequency was measured again  
39 by a second reviewer with less than a year of ICGL experience (\*\*). The video and  
40 vessel to study was shared between reviewers but measurements were performed  
41 independently.  
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## 51 Results

52 Sixteen healthy individuals aged between 20 and 55 years (7 females, mean age = 38.1  
53 years, median age = 37.5 years; 9 males, mean age = 39.4 years, median age = 42.9  
54 years) were recruited and imaged (see Supplementary Table 1). Lymphatic vessels  
55 actively transporting ICG towards the groin were seen in all imaged limbs, and no  
56 adverse effects were recorded during or after imaging.  
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## Lymphatic Anatomy

Contractile linear lymphatic vessels were observed in all 32 limbs of the enrolled participants (Fig 2). Vessels were traceable from injection site up to the groin in 87% of cases where thigh imaging was performed (27/31 limbs; in one limb thigh imaging was not performed). Regions of dermal backflow, lymph stasis and reflux were not observed. In one limb (3%), a vessel looped back on itself and pointed down the leg toward the foot before appearing to merge with another vessel travelling up the leg (Fig 3, Supplementary Video 1). In two limbs (6%), discontinuous lymphatic vessels were demonstrated, comprising seemingly unconnected vessel fragments. In a separate limb (3%) an unusually tortuous lymphatic vessel fragment was evident (Fig 4).

Anteromedial and anterolateral pathways were observed in 97% (31/32) and 78% (25/32) of limbs respectively (Fig 2), and all limbs had at least one, and at most 5, lymphatic vessels observed passing the anterior aspect of the ankle (mean  $\pm$  standard deviation =  $3.4 \pm 1.1$ ; median and mode = 4, interquartile range = 1).

In all but three cases (29 of 32 limbs) the posterior aspect of the leg (calf) was imaged. Posteromedial enhancement was noted in 18 limbs. Posterolateral pathways were only noted in 9 limbs, 7 of which also showed posteromedial vessels. Popliteal vessels were detected in 13 limbs, and a popliteal node was detected in 12 (Fig 5).

Of the 20 limbs where it was performed, in both limbs of the same participant was ICG manually drainable back down the limb suggestive of valve incompetence (Supplementary Video 2). This was not observed in any of the remaining 18 limbs.

Details of individual vessel pathways and vascular features can be found in Supplementary Table 1.

## Lymphatic Pumping

An average pumping frequency of  $0.9 \pm 0.4 \text{ min}^{-1}$  (range =  $0.0 - 2.0 \text{ min}^{-1}$ ) was estimated in anterior ankle lymphatics from videos lasting 180 – 250 seconds with 0 – 7 (mode and interquartile range = 2, median = 3) pumping events observed.

A subset of 10 videos was selected for repeat measurement by a second reviewer. Measurements obtained by the initial reviewer for these 10 resulted in an average frequency of  $0.9 \pm 0.5 \text{ min}^{-1}$  (range =  $0.0 - 1.4 \text{ min}^{-1}$ ), well matching the overall group mean. The second reviewer meanwhile recorded a pumping frequency of  $1.0 \pm 0.6 \text{ min}^{-1}$  (range =  $0.0 - 1.4 \text{ min}^{-1}$ ). An identical number of pumping events, and hence pumping frequency, was measured in 7/10 of the ICGL videos across reviewers. In the remaining

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4 3 videos, the second reviewer recorded -1, +1 and +2 pumping events when compared  
5 to the initial measurements.  
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## 7 8 Discussion 9

10 Indocyanine green (ICG) lymphography has emerged as an important technique for  
11 studying the superficial lymphatic system. It is particularly useful for studying vessels  
12 too small to observe with Magnetic Resonance Imaging (MRI) or lymphoscintigraphy  
13 (LS) and has been deployed in previously difficult regions to image such as the genitals  
14 (16–18). Its advantages are real time and in vivo visualisation of lymphatic transport  
15 with imaging devices that are relatively inexpensive and mobile. ICGL's clinical  
16 applicability is enhanced further by its minimally invasive and non-ionising nature.  
17 However, like any tracer-based technique, it is limited by possible contraindications to  
18 injection of a foreign agent and pain at administration. The poor penetrance of the  
19 infrared light also makes imaging vessels below around 2 cm in the skin impossible and  
20 the acquisition of image data is highly user dependent. Despite the limitations, ICGL has  
21 a role to play in the early detection and monitoring of lymphatic abnormalities, treatment  
22 planning and assessment (9,19,20).  
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29 Our recent review highlighted inconsistencies in reporting imaging details in ICGL  
30 studies and a lack of information regarding imaged participants however (10). Study  
31 protocols were also heterogenous, e.g. some authors report delaying imaging by  $\geq 1$   
32 hour after ICG injection whilst many image immediately such as we have here. ICG may  
33 also only be injected to the forefoot and neglect injections around the foot (10).  
34 Standardisation of injection and image acquisition protocols and common reporting  
35 guidelines, such as those developed by Suami et al. or Shinaoka et al. (9,21), may  
36 facilitate better comparison between studies and improve the diagnostic accuracy and  
37 clinical utility of ICGL (9,22).  
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43 In this observational study, we focused exclusively on the imaging of healthy subjects  
44 without a history of lymphatic dysfunction, attempting to establish normal lymphatic  
45 anatomy and function. Imaging healthy controls, and not the contralateral limb of  
46 unilaterally affected lymphoedema patients where subclinical disease may be present  
47 (12,23), is essential for establishing normative ICGL data, which will facilitate improved  
48 sensitivity in the detection of lymphoedema, particularly in its early stages.  
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51 We acknowledge that our sample size was limited ( $n=16$ ) but believe the broad age  
52 range and sex mix of the included participants has led to the generation of meaningful  
53 results within a control group and to which patient data could be compared.  
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## 56 Normal Lymphatic Drainage 57

58 The sites for administration of ICG were chosen to induce drainage via each of the four  
59 pathways described by Shinaoka et al. (14,15). While vessels from the anterior injection  
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4 sites, the anteromedial and anterolateral, were seen in all individuals, vessels following  
5 the posteromedial and posterolateral pathways were identified less frequently (18 and 9  
6 limbs respectively). Lymphatic vessels could be seen emanating from the posterior  
7 injection sites but often either followed a route more typical of the anteromedial and  
8 anterolateral lymphatics or were rapidly lost beyond the ankle. We suspect that the  
9 lower incidence of posterior lymphatic visualisation results either from these vessels  
10 residing deeper within the limb than their anterior counterparts or preferential drainage  
11 via anterior routes in healthy individuals.  
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16 Despite the limited penetrance of the technique, lymphatic vessels could generally be  
17 detected all the way to the groin in our cohort. Within the thigh and groin, substantial  
18 scattering of the emitted light was evident, and the vessels faint and blurred, likely a  
19 result of the deeper course of the lymphatic vessels in the thigh compared to the lower  
20 leg. Functional assessment of the lymphatics in the lower limb is therefore likely to be  
21 most sensitive in the leg (below the knee) as opposed to the thigh, and justifies our  
22 choice to assess valve competency, vessel count and pumping frequency in the ankle  
23 and leg region.  
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## 28 Incidental Findings and Interpretation 29

30 Although infrequent, some features potentially indicative of lymphatic abnormality were  
31 observed in our healthy cohort. These included segments of tortuous lymphatic vessels,  
32 lymphatic flow oriented down a limb, and suspected valve incompetence in which ICG  
33 containing lymph could be manually pushed back down the limb. In order to be included  
34 in this study, participants had to report no history of lymphatic disease, however we  
35 cannot rule out the presence of sub-clinical lymphatic abnormalities or previous  
36 undisclosed injury being the cause of these findings. We believe, however, natural  
37 variations in individual lymphatic systems (9,26,27) or limitations in the imaging  
38 technique (e.g. insufficient IR penetrance leading to a discontinuous lymphatic  
39 appearance when vessels travel from superficial to deeper and back toward the skin  
40 again) to be the most likely explanation for these infrequent findings.  
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47 Popliteal nodes were observed in 40% of our healthy cohort when the popliteal fossa  
48 was imaged. The presence of popliteal lymph nodes in lymphoscintigrams has been  
49 reported as suggesting pathological rerouting of the radionuclide via the deep  
50 lymphatics (24,25). Lymphoscintigraphy is typically preceded by injections only to the  
51 forefoot which would not be expected to access the lymphatics at the rear of the limb in  
52 a healthy individual, unlike the ICG injections at the rear of the foot performed in this  
53 study (14). Clinicians and researchers therefore need to be cognisant of the injection  
54 sites for any dyes or tracers used in imaging studies and whether popliteal  
55 enhancement should be considered abnormal.  
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## Quantitative Lymphatic Assessment

An ability to not only detect but also quantify lymphatic dysfunction is obviously desirable and has been performed with lymphoscintigraphy for some time by measuring the proportion of radioactive tracer reaching efferent lymph nodes following a delay post tracer administration (26–28). Lymphoscintigraphy lacks the temporal and spatial resolution to monitor lymphatic propulsion in real time, however. Measurement of lymphatic contractile frequency and the speed of ICG bolus movement in the lymphatics have previously been attempted in the arms of healthy controls (29,30), as has the arrival time of ICG at the groin of lower limb lymphoedema patients following ICG injections in the foot (31). This shows the potential use of ICGL as a tool for quantifying lymphatic transport but, to our knowledge, no report has established similar measurements for the lower limbs in healthy individuals. Establishing normal ranges for quantitative parameters such as these would be beneficial for improving the detection of lymphatic abnormalities, particularly those subtle changes which are not yet manifesting clinically (3).

Investigation of the anterior ankle enabled observation of linear vessels in all individuals, with 1 – 5 vessels visualised across all limbs. An average pumping rate of  $0.9 \text{ min}^{-1}$  was measured in the single vessel interrogated in each limb. This result is similar to that recorded in healthy forearms in the study of Lopera et al. where an average of  $0.9 \text{ min}^{-1}$  pumping events also resulted (30), and at the dorsum of the hands in a study of lymphoedema patients by Granoff et al. where  $1.1 \text{ min}^{-1}$  contractions were measured in the investigated vessels (32). Estimating pumping rate by eye was shown to be repeatable in this study, however processing pipelines have been proposed to improve the efficiency and robustness of monitoring lymphatic contractions with ICGL (29,30,32). Even within these reports where some level of automation has been employed, only single vessels were investigated, however. The development of more advanced bolus tracking and image post-processing pipelines, perhaps leveraging artificial intelligence, may facilitate the measurement of multiple vessels within a video frame in an acceptable time, and reduce the effects of lessened sensitivity and apparent resolution for those lymphatic vessels residing at the periphery of the image frame (29,32).

## Conclusion

Only by establishing normal parameters for anatomy, vessel numbers and characteristics of flow, can real pathological abnormalities be determined. ICGL is easy-to-use in the clinic and could be a valuable diagnostic tool if objective outcome measures, as developed in this study, are available. This would not only aid diagnosis of lymphoedema but help monitor disease progression. ICGL could also prove useful for the diagnosis of lymphatic abnormalities in previously understudied regions, such as the

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4 face, breast and genital regions, and improve patient management by demonstrating  
5 measured responses to therapeutic interventions. However, factors such as optical  
6 scatter, participant motion and restricted light penetrance, reduce the quality of ICG  
7 data and hence its interpretation.  
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10 Despite its limitations, healthy superficial lymphatic anatomy and functioning could be  
11 assessed in vivo and in real time in this study. Contractile superficial lymphatic vessels  
12 in each lower limb were observed in individuals with no history of lymphatic disease. We  
13 demonstrated that not all four lymphatic vessel groups of the lower limbs (anteromedial,  
14 anterolateral, posterolateral and posteromedial) will be observed in all cases, even with  
15 ICG injection sites chosen to access them. The lymphatic vessels which were observed  
16 were typically linear, with an average of 3 seen crossing the anterior ankle. Valve  
17 incompetency could be tested via the application of pressure down the limb and, along  
18 with tortuous lymphatic vessels, are generally absent in healthy legs. Tortuous vessels  
19 and valve reflux should therefore be considered abnormal, while depiction of popliteal  
20 lymphatic vessels and nodes should not when ICG is administered to the posterior of  
21 the foot.  
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## 30 Figure Captions

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33 Figure 1. The five ICG injection points in the foot (green dots) shown on a non-  
34 oedematous foot. The three frames show the injection locations of the medial rear-foot  
35 (left frame), 1st and 4th web spaces of the forefoot (middle frame) and the lateral midfoot  
36 and rear-foot (right frame). adapted with permission from (33) and subsequently  
37 published under a CC-BY 4.0 license in (10).  
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41 Figure 2. Representative Indocyanine Green Lymphography image of a 41-year-old  
42 healthy female's left lower limb. The location of injection sites can be seen at the bottom  
43 of the image, with the ICG traveling up toward the thigh at the top of the image. The  
44 anteromedial lymphatic vessels (highlighted with blue) originate from Indocyanine  
45 Green (ICG) injections in the 1st web space and 4th web spaces. Anterolateral  
46 lymphatic vessels (highlighted with green) can also be seen and originate from the ICG  
47 injection into the lateral midfoot. Four lymphatic vessels were observed passing the  
48 anterior aspect of the ankle (hashed box). Note that some lateral vessels move across  
49 to the medial limb just below the level of the knee (arrows).  
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54 Figure 3. Indocyanine Green Lymphography image of the ankle of a 51-year-old healthy  
55 female, in which a vessel is seen to loop back on itself before merging with another  
56 vessel travelling up the leg. There is therefore a region of reverse flow, where fluid  
57 motion is down toward the foot (direction of flow indicated by the purple arrow) and can  
58 be observed in Supplementary Video 1. The schematic on the right shows the  
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4 approximate location of the imaged region (hashed box) and the limb outline is shown in  
5 white.  
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8 Figure 4. Small tortuous vessel segment located at the medial calf in a healthy  
9 individual (51-year-old female, the same individual as shown in Figure 3). Lymphatic  
10 vessels with a curvilinear nature were commonly observed as they travelled up the limb,  
11 but such tightly tortuous vessels (purple arrow) were identified only in this one limb. As  
12 before, the schematic on the right shows the approximate location of the imaged region  
13 at the medial aspect of the leg (hashed box) and the limb outline is shown in white.  
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17 Figure 5. Posterolateral lymphatic vessel transporting Indocyanine Green from injection  
18 sites in the foot (unseen below frame) up the rear of the leg toward a lymph node  
19 (arrow) in the popliteal fossa of a 23-year-old male volunteer. No efferent vessels from  
20 the node into the thigh (upper portion of the image) could be observed. The schematic  
21 of the rear of the limb shows the approximate location of the imaged region (hashed  
22 box).  
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26 Supplementary Video 1. In a single limb, a vessel looped back on itself and pointed  
27 down the leg before appearing to merge with another vessel travelling up the leg (also  
28 shown in Figure 3). This was evidently the direction of flow for this vessel, and not  
29 reflux, as multiple pumping events were clearly observed in the vessel over the course  
30 of four minutes (30 second video shown for brevity). Interestingly, this individual also  
31 demonstrated a small tortuous lymphatic vessel segment, which is shown in Figure 4.  
32 Note that the orientation of this video matches Figure 3. in the main manuscript, with the  
33 superior aspect of the limb at the top of the frame and the inferior at the bottom. An  
34 exposure time of 40ms was employed when recording this video.  
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40 Supplementary Video 2. A 34 second clip of a 42-year-old female volunteer's leg in  
41 which lymph containing Indocyanine Green could be forced back down the limb (toward  
42 the top of the frame) by applying pressure to the limb. This suggests incompetent valves  
43 within this individual's lower limb lymphatics. Once the hand is removed, the lymphatic  
44 fluid rapidly flows back up the limb. A similar finding was noted for both lower limbs. A  
45 high exposure time (167ms) was required to visualise the lymphatics in this video  
46 reducing the temporal resolution to ~6 frames per second.  
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