SUPPLEMENTARY MATERIAL

SUPPLEMENTARY MATERIAL A: IN VITRO EXPERIMENTS

Near-Infrared Tracer

Near-Infrared Dye Characterization

QC-1, a near-infrared dye, (IRDye QC-1, LI-COR Biosciences, NE, USA; MW: 1.24 kDa) was used as a contrast agent for photoacoustic tomography (PAT). The lyophilized dye was reconstituted and diluted to different concentrations with 1x Phosphate Buffered Saline (PBS) for in vitro experiments. The absorbance of QC-1 dye at 25 µM was measured in duplicate samples from wavelengths 680nm to 980nm in 5nm steps using a spectrometer (Multi-Mode Reader Synergy Neo2 BioTek, VT) to obtain the absorbance spectrum of the dye (Fig. 1).

![Absorbance Spectrum](image)

**Fig. 1.** The absorbance (arbitrary units) of QC-1 dye at 25µM concentration from 680nm to 980nm in 5nm steps.

Photoacoustic Characterization of QC-1 Dye

To determine the relationship between QC-1 concentration and photoacoustic signal, phantoms with fourteen concentrations of QC-1 diluted in PBS (120, 80, 60, 40, 20, 10,
5, 2.5, 1.25, 0.625, 0.313, 0.156, 0.078, and 0.039 µM) were imaged using the MSOT inVision128 (iThera Medical Inc., Munich, Germany).

The phantoms were prepared using a protocol modified from Dean-Ben et al.’s protocol. 

1 Seven hundred and fifty mg of Agar (Sigma-Aldrich, St. Louis, MO) was mixed with 50 ml of deionized water to make a 1.5% mixture. The mixture was boiled in the microwave oven (Sanyo Electric, Osaka, Japan) and 2.5 mL of warm intralipid (20%, Sigma-Aldrich, MO) was immediately added to the mixture to make the final concentration of intralipid 1%. The agar-intralipid mixture was then poured into a cylindrical mold which was modified from a 20 ml syringe (Becton, Dickinson and Company, Franklin Lakes, NJ). Two capillary tubes (Thermo Fisher Scientific, ON, Canada) with an outer diameter of 1.5 mm and an inner diameter of 1.1 mm were placed symmetrically into the agar-intralipid mixture. After solidifying the phantom at room temperature, the capillary tubes and the external mold were removed. A new set of two sealed capillary tubes filled with a known concentration of QC-1 and vehicle (PBS, as a negative control) were then placed in the hardened phantom.

As the tracer injected into the anterior chamber was assumed to be completely mixed with aqueous humor, the estimated maximum concentration of the tracer *in vivo* was 120 µM (7 µL; Aqueous humor volume in CD1 mice2). Each concentration of QC-1 was imaged next to the negative control with vehicle (PBS) within the phantom with wavelengths 680 nm to 980 nm in 5 nm steps with 4 frame averages per wavelength, and at 6 different positions, 0.5 mm apart along the Z-axis. Each imaging session lasted approximately 5 minutes.
Photoacoustic images were processed offline and analyzed using the native viewMSOT software package (version 3.6; iThera Medical Inc.). Images were reconstructed using the backprojection algorithm. The reconstructed images were spectrally unmixed using the linear regression algorithm available within ViewMSOT using the 25 µM QC-1 absorbance spectra shown in Fig.1.3 In the spectrally unmixed images, cylindrical regions of interest (ROIs) with volume sizes of approximately 0.22 mm³ were taken from QC-1 and PBS samples within the 6 o’clock position of the capillary tube. The mean pixel intensity (MPI) and standard deviation were extracted from ROIs to assess the intensity of the signal. Linear regression analysis was performed between signal intensity and QC-1 concentration.

Quantification of the in vitro phantom photoacoustic images showed a linear relationship between signal intensity (MPI) and the QC-1 concentration from 0.078 µM to 120 µM (Fig. 2) (R² = 0.9273). The highest concentration of 120 µM was comparable to the final concentration of the dye after intracameral injection into the mouse eye (7 µL; aqueous humor volume).
SUPPLEMENTARY MATERIAL B: IN VIVO EXPERIMENTS

Near-Infrared Tracer Preparation - Conjugation of QC-1 Dye to BSA

For in vivo experiments, QC-1 dye was conjugated to Bovine Serum Albumin (BSA; Sigma-Aldrich, MO, USA; MW: 66.4 kDa) following the manufacturer’s protocol (IRDye 800CW Protein Labeling Kit-High MW, LI-COR Biosciences). The conjugate was concentrated using a centrifugal concentrator (Vivaspin Turbo 15, ON, Canada).

Measurement of QC-1/BSA Concentration and Dye/Protein Ratio

The QC-1/BSA conjugate was measured with a spectrometer (Multi-Mode Reader Synergy Neo2 BioTek, VT) to determine the concentration and labeling ratio. The absorbance of the tracer was measured at 280nm (the wavelength of maximum absorbance for the BSA protein) and 735nm (the closest laser setting to 737nm, the wavelength of maximum absorbance for QC-1) to calculate the dye/protein (D/P) ratio by the formula:

\[
\frac{D}{P} = \frac{A_{\text{max}}}{\varepsilon_{\text{Dye}}} = \frac{\frac{A_{\text{280}} - CF \cdot A_{\text{max}}}{\varepsilon_{\text{protein}}}}{A_{\text{735}}}
\]

\( \frac{D}{P} \): dye/protein ratio
\( A_{\text{max}} \): absorbance of the QC-1/BSA at QC-1’s maximum absorbance wavelength
\( A_{\text{280}} \): absorbance of the QC-1/BSA at 280nm
\( \varepsilon_{\text{Dye}} \): extinction coefficient of the QC-1
\( CF \): the correction factor (0.15 for QC-1)
\( \varepsilon_{\text{protein}} \): extinction coefficient of the BSA
The dye/protein ratio of QC-1/BSA was 1.88. The conjugate was diluted with PBS to obtain the BSA concentration at 10 mg/ml and the final QC-1 concentration was 280 µM.

**Intracameral Injection**

Under a surgical microscope (Zeiss OPMI 1, Carl Zeiss, Oberkochen, Germany), a right peripheral temporal corneal paracentesis was performed, releasing ~3 µL of aqueous humor prior to injection. Three µL of QC-1/BSA (280 µM) was injected intracameraly in the right eye (n=14; 7M/7F) with a 33 gauge needle on a 10 µL NanoFil syringe (World Precision Instruments, FL). The needle was removed and the cornea rinsed with artificial tear drops (GenTeal, Alcon, ON, Canada). This was followed by examination under the operating microscope for at least 10 minutes to exclude any failed injections due to hemorrhage, or other evidence of intraocular injury. The estimated concentration of the tracer in aqueous humor was 120 µM. Control experiments were performed to determine whether leakage during injection through the cornea may have contributed to tracer signal in the neck lymph nodes. PAT imaging was performed after topical application of 3 µL of QC-1/BSA (280 µM) using a microsyringe (n=4/F). Additional control experiments were performed to determine whether unconjugated QC-1 may contribute to the tracer signal in the neck lymph node. PAT imaging was performed after intracameral injection of 3 µL of QC-1 dye (without conjugation with BSA) (280 µM) (n=4/F) and compared with intracameral injection of QC-1/BSA (n=5/F).

**IOP Measurement**

The IOP of both eyes was measured using a rebound tonometer (TONOLAB, Icare Finland Oy, Vantaa, Finland) immediately before injection and after injection into the
right eye (n=10; 4M/6F). Measurements were taken by applying the probe perpendicular to the central corneal surface under a surgical microscope (Zeiss OPMI 1, Carl Zeiss, Oberkochen, Germany).

There was no statistically significant difference between mean IOPs of the right and left eyes before injection (15.22 ± 0.37 mmHg vs. 15.37 ± 0.50 mmHg; P = 0.7162; paired t-test). The mean IOP of the right eye decreased slightly showed no statistically significant difference between the right and left eye immediately after injection (9.85 ± 3.25 mmHg vs. 13.13 ± 2.25 mmHg, right and left eye, respectively; P = 0.77; paired t-test). IOP decrease after intracameral injection may be explained by isofluorane, and release of aqueous humor prior to injection (see above).

**Animal Preparation for In Vivo Imaging**

The day prior to imaging, the head and neck regions were gently shaved with a hair trimmer (ChroMini, WAHL, IL) under general anesthesia with 2% isoflurane and 100% oxygen. Hair removal cream (Nair, Church & Dwight, ON, Canada) was applied for 30 seconds to remove remaining hair. Just before the imaging, a thin layer of clear ultrasound gel (Aquasonic Clear, Parker Laboratories, NJ), warmed using a warmer (Thermasonic Gel Warmer, Parker Laboratories), was applied under general anesthesia. The eyes were kept moist with eye gel (GenTeal; Alcon, ON, Canada) during the experiment. Mice were placed prone in the animal holder, wrapped in 7 µm polyethylene foil (iThera Medical) in the mouse holder. Air bubbles present in the ultrasound gel between the mice skin and the foil were gently removed manually.

Each mouse underwent 5 imaging sessions (before, and 20 minutes, 2 hours, 4 hours, 6 hours after injection) and each imaging session lasted approximately 10 minutes. The
mice prepared for imaging were placed in the holder, which was connected to the anesthesia unit for general anesthesia with 2% isoflurane and 100% oxygen during the imaging session. The animal holder was mounted in the imaging chamber partially filled with warm water maintained at 34˚C. The mice were placed in the chamber for 3 minutes prior to each imaging session to allow for the body temperature to reach equilibrium. Photoacoustic scanning of the head and neck region was performed with eleven wavelengths: 680, 695, 735, 755, 775, 795, 825, 885, 925, 955 and 980 nm. Ten frames per wavelength were captured at each scanning position, and the step size between each scanning position was 0.5 mm. Each mouse was serially scanned from the eyes to the neck region (25 mm along the Z-axis and 51 positions in total), and each scanning session lasted approximately 10 minutes. After each imaging session, mice were disconnected from the anesthesia unit, and removed from the imaging chamber and the animal holder. The mice recovered from anesthesia after each imaging session in a clean cage placed over a heating pad (E-Z Anesthesia, PA) before being returned to the home cage with ad libitum food and water.

**Signal Visualization and Quantification**

Four out of 14 mice were excluded from analysis due to failed injections, anesthetic complications, or mechanical problems during imaging.

Photoacoustic images were reconstructed using the backprojection algorithm of the native viewMSOT software. The reconstructed images were spectrally unmixed using the adaptive matched filter algorithm (AMF) to visualize QC-1 signal compared to the images captured before tracer injection considered as the background.
Photoacoustic images were spectrally unmixed with the linear regression algorithm of the native software, with the QC-1 spectrum as well as oxyhemoglobin and deoxyhemoglobin selected as input spectra. The coordinates of 4 regions of interest (eyes and neck lymph nodes on right and left sides) were determined using the AMF-processed images described above and were transferred to the linear regression-processed images. The MPIs of QC-1 signal were extracted from each region to quantify signal.  

QC-1 MPIs were calculated for each of the 4 post-injection times, 20 minutes, 2, 4, and 6 hours and were transformed with the natural logarithm (logged MPI = ln(MPI + 100)). The logged MPIs were plotted over time for each mouse eye and node region, and a linear regression was performed. The slopes of the logged MPIs between 20 minutes and 6 hours post-injection were calculated. Additionally, the area under the curve (AUC) was calculated using the raw QC-1 MPIs from 20 minutes to 6 hours post-injection. The average slopes and the average AUCs were compared between the right and left corresponding ROIs using paired two-tailed t-tests. Statistical analyses were carried out with SAS (version 9.4, 2002-2012, SAS Institute Inc., Cary, NC), and GraphPad Prism (version 7.0c, GraphPad Software Inc., La Jolla, CA).

**In Vivo Control Experiments**

**Comparing Intracameral QC-1/BSA with Topical QC-1/BSA**

To determine whether tracer leakage during injections contributed to the signal in the neck lymph node, topical application of the QC-1/BSA was assessed. Three µL of QC-1/BSA (280 µM) was applied topically to the right cornea using a microsyringe (n=4/F).
In vivo PAT performed as described previously, detected minimal signal in the right eye at 20 minutes after application (Fig. 3). Photoacoustic images of neck area showed no signal in the right and left neck lymph nodes (Fig. 4).

To compare the differences between quantitative results following intracameral injection and topical application of QC-1/BSA, we used a linear mixed model with robust standard errors. A group by time interaction was used to test for the group difference in slope. If there was a significant interaction effect, we used Stata’s margins command to estimate and interpret the group differences. We calculated the area under the slope for each mouse by using the trapezoidal rule and completed a linear regression analysis on the derived values. We used margins to estimate and interpret group differences. All statistical analyses were completed using Stata 15.1 (College Station, TX).

Fig. 3. Photoacoustic images of coronal sections of the mouse head prior to (A), and after topical application of QC-1/BSA onto the right cornea (B-E). Prior to injection little to no tracer signal is observed (A). Twenty minutes after application, a small signal in green (arrow) is seen (B). However, no time points afterward show any signal (C, D, and E; 2, 4 and 6 hours after topical application, respectively). R (right), L (left), S (superior), I (inferior). Asterisks indicate the location of right eye when there is no signal. Arrow points to tracer signal in the right eye. The vertical green scale indicates tracer signal intensity (arbitrary unit). Scale bar = 3mm.
Fig. 4. Photoacoustic images of coronal sections of the mouse neck prior to (A), and after topical application of QC-1/BSA onto the right cornea (B, C, D, and E, (20 minutes, 2, 4 and 6 hours after topical application, respectively). Prior to injection, no signal is observed (A). No time points after application show any signal (B to E). R (right), L (left), S (superior), I (inferior). Asterisks indicate the location of right neck lymph node without any tracer signal. The vertical green scale indicates tracer signal intensity (arbitrary unit). Scale bar 3mm.
As shown above in Fig. 5, the mean slope of the QC-1 signal in the right eye after intracameral QC-1/BSA in the right eye was significantly steeper than that observed after topical QC-1/BSA in the right eye (P=0.0001). The mean AUC of the QC-1 signal after intracameral QC-1/BSA in the right eye was significantly larger than that observed after topical QC-1/BSA in the right eye (P=0.0001).
As shown above in Fig. 6, the mean slope of the QC-1 signal in the right neck lymph node after intracameral QC-1/BSA in the right eye was significantly steeper than that observed after topical QC-1/BSA in the right eye (P=0.0004). The mean AUC of the QC-1 signal in the right neck lymph after intracameral QC-1/BSA in the right eye was significantly larger than that observed after topical application of QC-1/BSA in the right eye (P=0.0003).
Comparing Intracameral QC-1/BSA Injection with Intracameral QC-1 Injection

To determine whether unconjugated QC-1 might contribute to detected signal in the neck lymph node, QC-1 (without BSA conjugation) was injected into the right anterior chamber, and PAT imaging was performed before and after intracameral injection of QC-1 (n=4/F). These results were compared with those obtained after intracameral injection of QC-1/BSA using statistical tests as described above. The detection of tracer signal from the right eye is shown in Fig. 7. Fig. 8 shows photoacoustic images of the neck region.
Fig. 7. Photoacoustic images of coronal sections of the mouse head prior to, and after injection of free QC-1 (without BSA conjugation) into the right anterior chamber. Prior to injection, no signal is observed (A). Twenty minutes after injection, a bright signal in green is seen (B). The signal decreased over 6 hours (C, D, and E, 2, 4 and 6 hours, respectively). R (right), L (left), S (superior), I (inferior). Asterisk indicates the location of the right eye without signal in A. Arrows point to tracer signal in the right eye. The vertical green scale on the right indicates QC-1 signal intensity (arbitrary unit). Scale bar = 3mm.

Fig. 8. Photoacoustic images of coronal sections of the mouse neck prior to, and after injection of free QC-1 (nonconjugated with BSA) into the right anterior chamber. Prior to injection, no signal is observed (A). No signal is detected after the injection (B, C, D and E, 20 minutes, 2, 4, and 6 hours after injection, respectively). R (right), L (left), S (superior), I (inferior). Asterisks indicate the location of right neck lymph node when there is no signal. The vertical green scale on the right indicates QC-1 signal intensity (arbitrary unit). Scale bar = 3mm.
Fig. 9. The average photoacoustic signal in the right eye over 6 hours for CD-1 mice after intracameral injection of QC-1/BSA (n = 5/F). Red and gray circles show the right and left eye, respectively. Also shown, is the average photoacoustic signal in the right eye over 6 hours for CD-1 mice after intracameral injection of QC-1 only (n=4/F) into the right eye. Blue and orange circles show the right and left eye, respectively. Error bars indicate standard deviation.

As shown in Fig. 9, the mean slope of the tracer signal in the right eye after intracameral QC-1/BSA in the right eye was significantly steeper than that observed after intracameral QC-1 in the right eye (P=0.0106). The mean AUC of the QC-1 signal after intracameral QC-1/BSA in the right eye was significantly larger than that observed after intracameral injection of QC-1 in the right eye (P=0.0169).
As shown above in Fig. 10, the mean slope in the right neck lymph node after intracameral QC-1/BSA in the right eye was significantly steeper compared to QC-1 injected in the right eye (P=0.0004). The mean AUC of the signal in the right neck lymph node after intracameral QC-1/BSA was significantly larger compared to QC-1 injected in the right eye (P=0.0003).
SUPPLEMENTARY MATERIAL C: EX VIVO VALIDATION

Near-Infrared Fluorescent Tracer

Near-infrared fluorescent dye, CF770 (Biotium, Fremont, CA; MW 3.14 kDa) was used for ex vivo fluorescence optical imaging. CF770 was conjugated with BSA, a protein fixable with paraformaldehyde as described above. The dye/protein ratio of CF770/BSA was 2.45, and CF770 concentration was 1.4 mM as determined using the method described for QC-1 (Supplementary Material A; Equation 1) at 770 nm, the maximum absorption wavelength of CF770. The correction factor for CF770 was 0.06 according to the manufacturer's instructions.

Tracer stability: Lymph Node Protein Electrophoresis

To check the stability of near-infrared dye-labeled BSA, sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) of lymph node homogenates was performed. Three µL of CF770/BSA was injected into the right eye as described above. Mice were sacrificed 4 hours after injection. Right and left submandibular lymph nodes were dissected and flash frozen in liquid nitrogen separately. The whole lymph node was homogenized in 50 µl of NP-40 lysis buffer (150 mM sodium chloride (Bioshop, ON, Canada), 1% NP-40 (Sigma-Aldrich, MO, USA), 50 mM Tris (Bioshop, ON, Canada) pH 8.0) with 0.1% SDS (Sigma-Aldrich, MO, USA) on ice. Homogenates were centrifuged (Eppendorf, Hamburg, Germany) at 12,000 rpm for 10 min at 4 °C. The whole supernatant (approximately 40 ul) was taken out and mixed with 10 ul of 5X Lamelli Buffer (60 mM Tris-Cl pH 6.8, 2% SDS, 10% glycerol (Thermo Fisher Scientific, ON, Canada), 5% β- mercaptoethanol (Sigma-Aldrich, MO, USA), 0.01% bromophenol blue (Sigma-Aldrich, MO)). The mixture was heated up to 95 °C for 5 min before being loaded on a 10% Tris-
Glycine gel. Five nanograms of CF770/BSA were loaded as a control and 2 µl of BLUeye prestained protein ladder (FroggaBio, ON, Canada) was used as a molecular weight indicator. Electrophoresis was carried out with the Mini-Protean electrophoresis system (Bio-Rad Laboratories, CA, USA) at a constant voltage (110V) for 2 hours. After protein ladder was marked with 10nM of CF770 on the glass plate, the whole gel with glass plates was imaged using the LI-COR Odyssey Fc Imaging System (LI-COR Biosciences, NE, USA) (Fig. 11). Homogenates of the right submandibular node 4 hours after injection of CF770/BSA showed a strong signal by SDS PAGE with intact size compared to CF770/BSA derived from freshly prepared stock (Fig. 11).
Ex Vivo In Situ Optical Fluorescence Imaging After Intracameral Injection of CF770/BSA

To map the head and neck lymph nodes draining intracameraly injected tracer, CF770/BSA was injected intracameraly in the right eye under general anesthesia as

Fig. 11. Protein electrophoresis of lymph node homogenates after right injection of 3 μL of CF770/BSA. Lane 1: protein ladder; Lane 2: 5ng of CF770/BSA tracer from freshly prepared stock as control; Lane 3: protein extracted from right submandibular lymph node; Lane 4: protein extracted from left submandibular lymph node. Electrophoresis experiments were repeated 3 times with different mice.
described above (n=3). Mice were sacrificed 4 hours after injection and dissected carefully to remove the skin of head and neck area as well as the fat tissue. Images of neck lymph nodes were taken using a digital camera (iPhone 6, Apple, CA, USA) under a surgical microscope (Fig. 12). Detailed study of the lymph nodes was performed based on previous anatomical descriptions of lymph nodes in mice.9,10 The head and neck region including the submandibular glands was imaged using a confocal scanning laser ophthalmoscopy system (Spectralis, Heidelberg Engineering, Heidelberg, Germany). Blue Autofluorescence (BAF) (λ=486nm) scans were used to distinguish lymph nodes and glands after intracameral injection of CF770/BSA. Near-infrared Fluorescence (NIRF) (λ=786nm) scans were used to detect the near-infrared fluorescence signal. All the images were taken under the same settings. Submandibular glands and related superficial lymph nodes were removed after imaging to expose the deep cervical lymph nodes (Fig. 12). Near-infrared fluorescence scans detected strong signal in the right submandibular lymph node, right accessory submandibular lymph node and right deep cervical lymph node (Fig. 13).
Fig. 12. Cervical lymph nodes in the CD1 mouse. Ventral view of mouse neck and head region after removal of skin (A). Some of the superficial cervical lymph nodes (arrows) could be identified but were covered with fat tissue. After careful dissection, fat and parotid glands were removed, 3 pairs of lymph nodes (arrows) (1: submandibular; 2: accessory submandibular; 3: superficial parotid) were clearly observed (B). In a deeper plane, after removal of submandibular glands and superficial cervical lymph nodes, right and left deep cervical lymph nodes (4) at either side of the trachea were revealed (C). R: right side; L: left side; SG: submandibular glands; Tr: trachea. Scale bar indicates 2 mm.
Fig. 13. Ex vivo optical fluorescence imaging after intracameral injection of tracer: Ventral view of the mouse head and neck region 4 hours after right eye intracameral injection of CF770/BSA. After skin and fat removal, blue autofluorescence (BAF) scan (A) showed submandibular gland (SG) and submandibular lymph nodes (1), accessory submandibular lymph nodes (2), and superficial parotid lymph nodes (3). NIRF scans (B) detected strong tracer signal in the right side lymph nodes. Merged image (C) of BAF (A) and NIRF (B) images confirmed the presence of tracer signal (green) in the right submandibular lymph node, right accessory submandibular lymph node. BAF scan of the deep plane after removal of superficial nodes and glands (D) showed deep cervical lymph nodes (4) at either side of the trachea. NIRF scan (D) detected strong tracer signal in the right deep cervical lymph node. Merged image (F) of BAF (D) and NIRF (E) images confirmed the presence of tracer signal (green) confirmed the presence of tracer signal in the right deep cervical lymph node. R: right side; L: left side; Ant: anterior; Tr: trachea. Scale bar, 1 mm.

**Ex Vivo In Situ Fluorescence Imaging After Subconjunctival Injection of CF770/BSA**

Three µL of CF770/BSA was injected into the temporal subconjunctival space of the right eye (n=2) to determine the possible role of conjunctival lymphatics in the local drainage of intracameraly injected tracer and to assess whether the conjunctival lymphatics drain into the same lymph nodes as observed after intracameral injection of...
the tracer. BAF scans showed the lymph nodes (arrow) and glands (SG). NIRF scans detected strong signal in the right submandibular lymph node, right accessory submandibular lymph node, right superficial parotid node and right deep cervical lymph node 4 hours after CF770/BSA subconjunctival injection with minimal tracer signal in left side neck lymph nodes (Fig. 14). Table 1 summarizes ex vivo fluorescence imaging data.

Fig. 14. Ex vivo optical fluorescence imaging after subconjunctival injection of tracer. Ventral view of the mouse head and neck region 4 hours after right eye subconjunctival injection of CF770/BSA after removal of skin and fat: BAF scan (A) showed submandibular gland (SG) and submandibular lymph nodes (1), accessory submandibular lymph nodes (2), and superficial parotid lymph nodes (3). NIRF scans (B), detected strong tracer signal in the right side lymph nodes. Merged image (C) of BAF (A) and NIRF (B) images confirmed the presence of tracer signal (green) in the right submandibular lymph node, right accessory submandibular lymph node, and right superficial parotid node. Blue autofluorescence scan of the deep plane after removal of superficial nodes and glands (D) showed deep cervical lymph nodes (4) at either side of the trachea. Near-infrared fluorescence scan (E) detected strong tracer signal in the right deep cervical lymph node. Merged image (F) of BAF (D) and NIRF (E) images confirmed the presence of tracer signal (green) in the right deep cervical lymph node. A smaller amount of tracer signal was noted in the left deep cervical lymph node. R: right side; L: left side; Ant: anterior; Tr: trachea. Scale bar, 1 mm.
Table 1. Near-infrared fluorescent tracer (CF770/BSA) signal in cervical lymph nodes (LN) after intracameral and subconjunctival injections

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<th>Intracameral CF770/BSA</th>
<th>Subconjunctival CF770/BSA</th>
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<td><strong>Accessory submandibular LN</strong>s</td>
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<td><strong>Superficial parotid LN</strong>s</td>
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<td><strong>Deep cervical LN</strong>s</td>
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Data are LN containing CF770 fluorescent signal (N positive/N tested)

**Histopathological Validation**

Near-infrared fluorescent dye, CF770 (Biotium, Fremont, CA; MW: 3.14 kDa) was injected into the right anterior chamber of 4 mice. CF770 was conjugated with BSA, a protein fixable with paraformaldehyde. The dye/protein ratio of CF770/BSA was 2.45, and CF770 concentration was 1.4 mM as determined using the method described for QC-1 (Supplementary Material B) using the absorbance at wavelength 770nm, the maximum absorption wavelength of CF770. Three µL of CF770/BSA was injected in the right eye of 4 mice as described for QC-1/BSA injections (Supplementary Material B).
and mice were sacrificed 2 hours (n=2) and 4 hours (n=2) later. BSA without dye was injected intracamerally into the right eye of 2 mice as negative controls and they were sacrificed 6 hours later.

**Lymph Node Tissue Preparation and Sectioning**

Sacrifice was performed by transcardial perfusion with 20ml of chilled PBS followed with 20ml of chilled 2% paraformaldehyde (Electron Microscopy Sciences, PA) under general anesthesia with 2% isoflurane and 100% oxygen. Neck soft tissue blocks and a tissue block containing the left inguinal node were harvested and immersion-fixed in 2% paraformaldehyde for 16 hours at 4˚C followed by three times of PBS rinse at room temperature. The specimens were then cryoprotected by immersion in 10% sucrose (Thermo Fisher Scientific) in PBS for 24 hours and 20% sucrose in PBS for 48 hours at 4˚C. The blocks were frozen in isopentane (Thermo Fisher Scientific) cooled by dry ice and embedded into cryomatrix (Tissue-Tek O.C.T. Compound, Sakura, South Holland, Netherlands) and serially sectioned (40 µm thick) using a cryostat (Leica CM1900, Leica Biosystems, ON, Canada). Sections were mounted onto charged microscope slides (Superfrost Plus, Thermo Fisher Scientific).

**Immunofluorescence Staining of Lymph Node Sections**

The sections were washed three times in PBS for 5 minutes each and incubated for 1 hour with 2% goat serum (Sigma-Aldrich, MO, USA) and 0.2% Triton X-100 (Sigma-Aldrich, MO, USA) in PBS. Sections were then incubated with collagen IV antibody, a basement membrane marker, (1:400; Rabbit polyclonal, Abcam, ON, Canada) with 2% goat serum and 0.2% Triton X-100 in PBS overnight at 4˚C. After three 10 minutes PBS washes, sections were incubated with goat anti-mouse Alexa Fluor 647 secondary
antibody (1:1000; Thermo Fisher Scientific, ON, Canada) with 2% goat serum and 0.2% Triton X-100 in PBS for 1 hour in the dark. The sections were counterstained with a nuclear stain (Sytox Green; 1:2000; Thermo Fisher Scientific). After three washes in PBS for 10 minutes each, the sections were covered with a coverslip (#1.5, Thermo Fisher Scientific) and aqueous mounting medium (Dako, Agilent Technologies, CA). All sections were treated at room temperature with mild agitation unless otherwise noted.

Near-Infrared Fluorescence Microscopic Imaging of Lymph Node Sections

Sections were imaged using an upright fluorescence microscope (BX51, Olympus, Tokyo, Japan) equipped with a custom-designed Image Mapping Spectrometer (IMS) snapshot hyperspectral scientific CMOS camera\(^{11}\) (pco.edge 5.5, PCO AG, Germany). The IMS sampled hyperspectral images at 78 wavelengths from 528 to 836 nm in 4 nm steps. The sections were illuminated with an external Xenon light source (Lambda LS 175W, Sutter Instrument Company, CA) and imaged with a 10x objective (Olympus). Fluorescence filter cubes used were GFP Longpass (19002, Chroma Technology Corporation, VT) for nuclear staining, Cy5 (U-N41008, Olympus) modified with a longpass emission filter (ET665lp, Chroma) for Alexa Fluor 647, and IRDye800 (49037 - ET - Li-Cor for IR dye 800, Chroma) for CF770.

A custom-designed software (Reveal™ v1.4, Apre Instruments LLC., Tucson, AZ) was used to interface with the IMS and a motorized stage (BioPrecision MAC5000, Ludl Electronic Products Ltd., NY). The imaging was performed with two averaged acquisitions per frame at exposures of 250, 500, and 1500 ms for GFP, Cy5, and IRDye800 channels, respectively. A predefined area of the tissue was selected and automatically captured with multiple overlapping fields of view with the computer-
controlled motorized stage. Uniformly fluorescent calibration slides (92001, Chroma) and dark noise images were captured as 32 frame averages, to flat-field correct the sample images.\textsuperscript{12} Raw images were saved in HDF5 format, along with stage coordinates. Images were processed with ENVI v5.4 (Harris Corporation, Boulder, CO). The pixel size was measured using a square grid calibration slide (MBF Bioscience, VT). Individual images were coregistered using stage coordinates and pixel size. A flat-field correction was applied by previously described methods.\textsuperscript{12} Overlapping co-registered images were then joined to form one large mosaic image using the Seamless Mosaic Toolbox (ENVI v5.4). Individual wavelengths of the hyperspectral data were used to create pseudo-color composite images. Blue (Sytox Green) = 540 nm, green (CF770/BSA) = 780 nm, and red (collagen IV immunostain with AlexaFluor 647) = 680 nm. Mosaics were thresholded to the same values and exported in TIFF format. Scale bars were inserted using ImageJ 1.51j (National Institutes of Health, Bethesda, MD).
REFERENCES