CHAPTER

14 Optimizing Fluorescence Excitation and Detection for Intravital Two-Photon Microscopy

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CHAPTER OUTLINE

Abstract

Commercial two-photon microscope systems incorporating turnkey ultrafast lasers have made the technology more user-friendly and accessible to nonspecialized biology laboratories. This has been accompanied by the development of an exciting

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range of new fluorescent proteins and dyes such as near-infra-red fluorescent proteins and optical highlighters. However, the two-photon absorption properties of these fluorescent molecules are not widely available and cannot be reliably predicted from their single photon absorption spectra. Furthermore, the spectral characteristics of fluorescent proteins *in vivo* can be affected by the local environment and light scattering by deep tissue and can vary greatly from one laboratory to the next. Here, we describe a simple protocol for determining the two-photon excitation peaks of fluorescent reporters that can be tailored to the relevant tissue samples to suit the imaging goals of individual biological laboratories.

1 PURPOSE

Two-photon excitation cross-sections are often measured by physics laboratories and can vary considerably depending on the method and laboratory. Therefore, it is imperative that biologists optimize the fluorescence excitation for the conditions under which they typically perform their experiments. The purpose of this chapter is to provide practical tips and guidelines for biologists to acquire excitation spectral fingerprints for intravital two-photon microscopy in deep tissue. The protocol can be easily performed with most commercially available two-photon microscope systems equipped with a software-tunable ultrafast near IR laser and electronic laser intensity control. This will allow the two-photon fluorescence excitation to be optimized for the biology under the same conditions as the actual imaging experiments.

2 THEORY

Two-photon microscopy provides an ideal platform to image dynamic biological processes in deep tissue of live animals with minimal perturbation to the underlying physiology (Helmchen & Denk, 2005; Zipfel, Williams, & Webb, 2003). Recent improvements in ultrafast laser technology have made it possible for biologists to operate two-photon microscopes without specialized training in laser physics. Practical guidelines are therefore needed to facilitate the uptake and application of two-photon microscopy to answer basic biological questions (Phan & Bullen, 2010). General principles and protocols for instrument setup, sample preparation, image acquisition and data analysis have been described in detail in a number of excellent published methods (Kitano & Okada, 2012; Matheu, Cahalan, & Parker, 2011; Zinselmeyer et al., 2009). To take advantage of the increasing range of fluorescent proteins and dyes for multicolor two-photon imaging, we will therefore focus on the steps required to acquire a two-photon excitation fingerprint and thereby optimize the choice of fluorophores and excitation wavelengths for different imaging experiments. These new reporters include, but are not limited to, the

recently described infra-red fluorescent proteins (Shu et al., 2009) and optical highlighters (Patterson, 2011) such as Kaede (Ando, Hama, Yamamoto-Hino, Mizuno, & Miyawaki, 2002) and KikGR (Tsutsui, Karasawa, Shimizu, Nukina, & Miyawaki, 2005).

Two-photon excitation is a nonlinear process and therefore the excitation spectra cannot be entirely predicted from the single photon excitation spectra. In general, the excitation peaks are shifted to shorter wavelengths than would be expected by just doubling the wavelength and are often much broader (Zipfel et al., 2003). In addition, the relative intensities of the peaks may vary (Drobizhev, Makarov, Tillo, Hughes, & Rebane, 2011). However, while this applies to some classes of fluorescent proteins such as eGFP, it does not apply to fluorescent dyes such as Alexa Fluor 488 (Dickinson, Simbuerger, Zimmermann, Waters, & Fraser, 2003). Unfortunately, two-photon absorption cross-sections are not readily available for a number of fluorescent proteins. Furthermore, there is considerable variability in the two-photon cross-section of fluorescent proteins measured by different methods in different biophysics laboratories (Drobizhev et al., 2011). Finally, the local microenvironment may impact on the fluorescent properties of fluorophores, for example, through electrostatic interactions, protein aggregation and changes in pH. Thus, it is highly likely that the two-photon excitation fingerprint of a fluorophore in aqueous solution or embedded in agarose will differ from that in biological samples. In addition, the imaging depth of a typical experiment must be taken into account as shorter wavelengths will not penetrate as far as longer wavelengths. Therefore, it is critical for investigators to acquire excitation lambda stacks for each fluorescent protein and dye to optimize the two-photon excitation wavelengths for their particular experimental models and conditions (Dickinson et al., 2003).

Excitation lambda scans can be performed with ultrafast software-tunable IR lasers with electronic laser intensity control devices. They differ from emission lambda scans in that the laser excitation wavelength is tuned automatically while the fluorescence output is detected with a single non-descanned detector (NDD) with the relevant filter. In contrast, emission fingerprints are performed by excitation with a single laser wavelength and scanning the resulting fluorescence emission across a number of wavelength bandwidths (for example, by using a multichannel spectral detector). Excitation fingerprints obtained with different detection channels can be used to determine the level of cross-talk in the same way emission fingerprints are used to perform linear spectral unmixing (Dickinson et al., 2003). More importantly, it can be used to guide the choice of fluorophores and excitation wavelengths for complex multicolor experiments involving multiple fluorescent tags.

3 EQUIPMENTS

Below is a list of the equipments needed to perform excitation lambda scans and the key points to look for.

3.1 Software-Tunable Ultrafast Infra-Red (IR) Laser

We use a Chameleon Vision II laser (Coherent Scientific) with a peak power output of 3.6 W at 800 nm, which can tune from 680 to 1080 nm at a rate of 40 nm/s. The laser has integrated dispersion precompensation (prechirp). The laser wavelength and dispersion compensation settings are controlled by the real-time computer which also controls and synchronizes data acquisition by the microscope.

3.2 Two-Photon Microscope with Electronic Laser Intensity Control

We use an upright Zeiss 7MP two-photon microscope (Carl Zeiss) with a W Plan-Apochromat 20×/1.0 DIC (UV) Vis–IR water immersion objective. Excitation fingerprints were performed with an LBF 690 short pass IR blocking filter and BSMP 690 dichroic for the NDD sideport. Four external NDDs are used to detect blue (SP 485), green (BP 500–550), red (BP 565–610) and far red (BP 640–710) emissions. The laser beam is coupled to an acousto-optical modulator (AOM), which provides precise real-time electronic control of the laser power intensity. Mechanical laser intensity control devices such as half-wave plates and neutral density filters are too slow and imprecise for excitation fingerprinting. The laser, microscope, AOM, laser scanner and peripherals in the 7MP are controlled by the real-time computer. We use the ZEN software interface to perform lambda scans using a macro function.

3.3 Light Meter

We use a Fieldmaster (Coherent Scientific) portable power/energy meter to measure the laser power intensity at the objective. This is used to generate the laser calibration curve needed to perform the excitation lambda scan.

3.4 Dissecting Instruments

Dissecting scissors and forceps to harvest organs and tissues.

3.5 Stereomicroscope

We use a Stemi 2000 stereomicroscope (Carl Zeiss) with a zoom factor of 7 illuminated by a CL 1500 cold light source with a conversion filter.

3.6 Cold Light Source with Output in the Violet Range

This is used to photoconvert the KikGR from green to red.

3.7 Imaging Chamber

We use a JG-23 ultraquiet imaging chamber (Warner Instruments).

4 MATERIALS

Fluorescent reporter mice: There are a number of genetically encoded fluorescent reporter mice that can be used for two-photon microscopy. In this protocol, we will describe the two-photon excitation properties of green and red species of the optical highlighter KikGR in a novel genetically modified mouse line established by Kanagawa and Tomura (Tomura et al., manuscript in preparation).
RPMI
VetBond tissue glue
Silicone grease

5 PROTOCOL

Duration

	Time
Preparation	1 h
Protocol	25–50 min

Preparation Clean and sterilize surgical instruments.
Caution Class 4 laser safety.
Tip Laser power calibration curves can be performed in advance and stored until required for the excitation lambda scan.

See the flowchart in Fig. 1.

5.1 Step 1—Laser Power Calibration

Overview Laser power intensity measurements at the objective provide an indication of the actual laser power in the biological tissues during imaging. Measurements should be performed routinely every month as part of the quality assurance system. The measured intensity can also be used to construct a laser power calibration curve to perform excitation lambda scans.

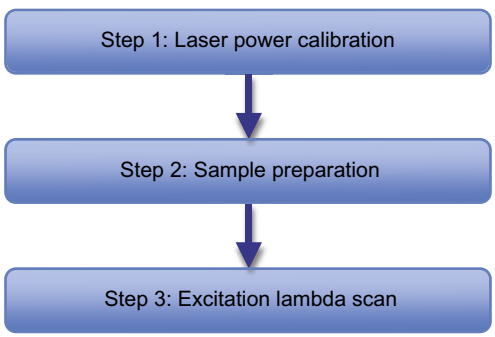

FIGURE 1

Flowchart showing steps involved in the protocol. For color version of this figure, the reader is referred to the online version of this book.

Duration	5–10 min.
1.1	Turn on laser.
1.2	Turn on microscope.
1.3	Turn on acquisition computer and launch acquisition software.
1.4	Turn PMT gain on all NDDs down to zero. If GAsP detectors are installed, leave these off.
1.5	Position probe of light power meter immediately under the objective. Be sure to use the same position each time.
1.6	Set the AOM to attenuate the laser to 5%.
1.7	Set the microscope to 512 × 512 pixel bidirectional scan at 10 frames/s (1.27 μs per pixel).
1.8	Set the zoom factor to 40.
1.9	Tune laser to 690 nm.
1.10	Record laser power intensity at objective with light power meter.
1.11	Increase the wavelength in 10 nm bandwidths and record laser power until 1060 nm.
1.12	Plot the laser power curve in Excel or Prism (see Fig. 2).
1.13	Calculate the AOM laser attenuation required to produce 20 mW of laser power at the objective.
1.14	Open the excitation fingerprinting macro in ZEN software.
1.15	Enter the AOM laser attenuation for each wavelength and save the laser calibration curve.
Caution	Ultrafast IR lasers are class 4 laser hazards. Ensure all relevant OHS training and precautions are undertaken. Do not measure the laser power without significant levels of attenuation. Shield the microscope stage and use laser safety goggles to minimize radiation exposure.

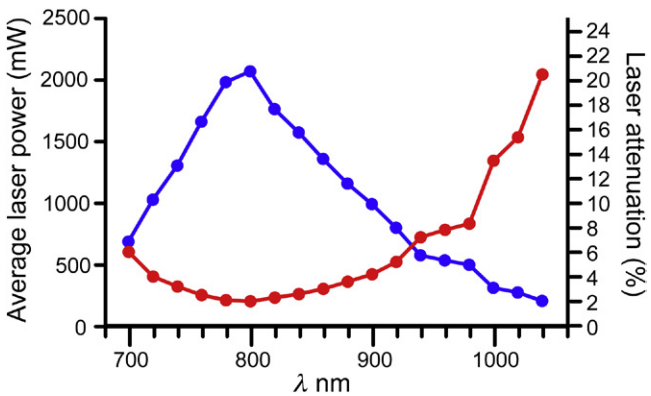

FIGURE 2

Laser power calibration curve. The laser output was attenuated to 5% and the laser power intensity at the objective was measured with a light power meter from 690 to 1060 nm. The average of three readings was used to calculate the laser power without attenuation (blue line, left axis). The AOM attenuation required to produce the same laser power at different wavelengths was then calculated (red line, right axis). (See color plate.)

Tip Laser power intensities for the commonly used wavelengths (e.g. 810 nm and 920 nm) can be plotted and tracked on a Levey-Jennings chart to detect shifts and drifts that may indicate problems with the laser or laser alignment.

See Fig. 3 for the flowchart of Step 1.

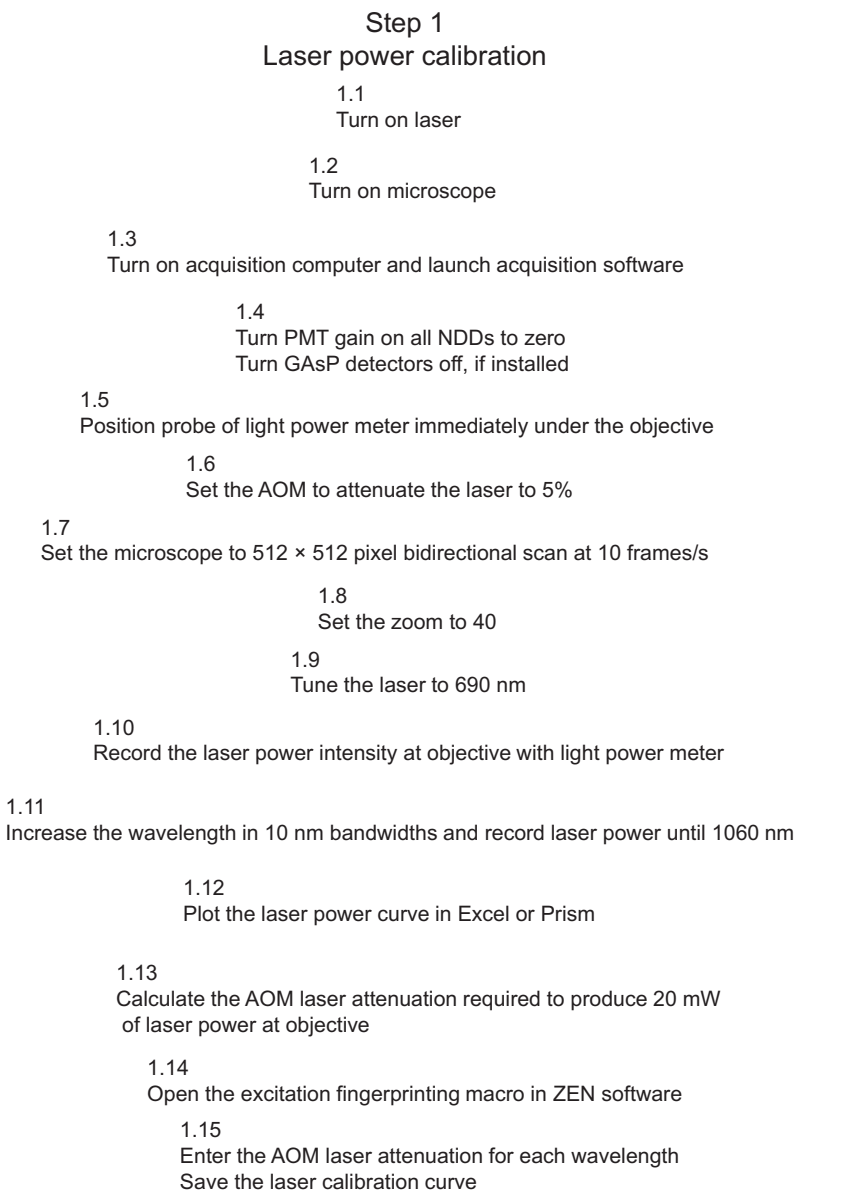

FIGURE 3

Flowchart of Step 1.

5.2 Step 2—Sample Preparation

Overview	The relevant tissue should be prepared as per the laboratory protocol for two-photon imaging to ensure that the excitation fingerprint is applicable to the biology in question. Here, we describe preparation of mouse lymph node for explant imaging. More details and online videos demonstrating the technique can be found in the *Journal of Visual Experiments* (Matheu, Parker, & Cahalan, 2007). Excitation lambda scans can be performed rapidly after sample preparation and do not require preservation of cell motility. Therefore, it is sufficient to immerse the lymph node with tissue culture media at room temperature.
Duration	15–30 min.
2.1	Sacrifice the mouse and harvest lymph node.
2.2	Prepare lymph node for two-photon imaging by carefully removing any fat and connective tissue under the stereomicroscope.
2.3	Gently mount the lymph node on a plastic coverslip with VetBond tissue glue.
2.4	Remove excess tissue glue under the stereomicroscope.
2.5	Use silicone grease to adhere the coverslip to the imaging chamber.
2.6	Immerse the lymph node in RPMI for imaging.
Caution	Use only very small amounts of VetBond as it is fluorescent and can interfere with imaging.
Tip	Careful dissection of the lymph node from the mouse along fascial planes will remove most fat and connective tissue and speed up the cleaning step. Ensure all excess glue is removed.

See Fig. 4 for the flowchart of Step 2.

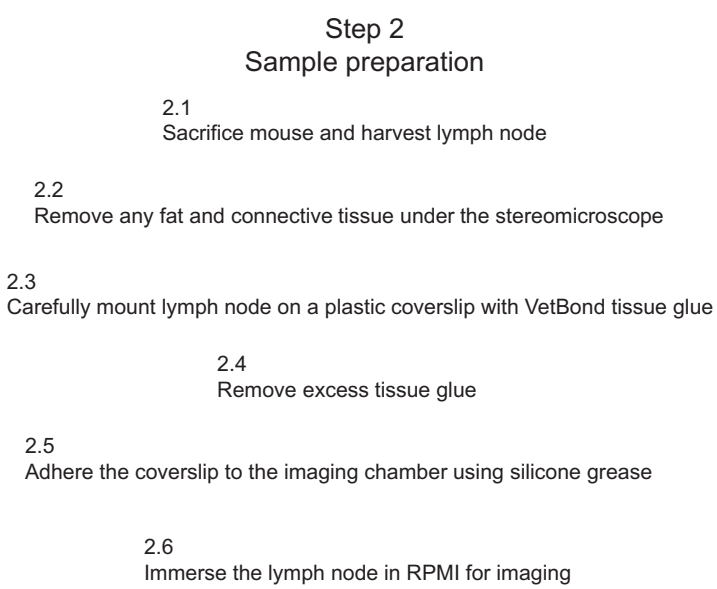

FIGURE 4

Flowchart of Step 2.

5.3 Step 3—Excitation Lambda Scan

Overview	In this step, we will use the preloaded macro in a commercial two-photon system to automatically acquire an excitation lambda stack. However, this step can be performed manually in any two-photon microscope with a software-tunable IR laser and electronic laser intensity control device.
Duration	5–10 min.
3.1	Open the excitation fingerprinting macro.
3.2	Load the laser calibration curve from Step 1.
3.3	Enter the range of wavelengths for excitation lambda stack from 700 to 1040 nm.
3.4	Position the unphotoconverted lymph node from KikGR mouse in the imaging chamber under the objective.
3.5	Locate the surface of the lymph node by the second harmonic signal from collagen in the capsule at 810 nm.
3.6	Move the objective focal plane 100 μm below the capsule. This is typical of the depth we image at.
3.7	Turn on green NDD.
3.8	Acquire excitation lambda stack for green species of KikGR in the green fluorescence channel.
3.9	Turn off green and turn on red NDD.
3.10	Acquire excitation lambda stack for green species of KikGR in the red fluorescence channel.
3.11	With the lymph node *in situ* on the microscope stage, irradiate it with violet light from cold light source to optimally photoconvert the lymph node.
3.12	Turn on green NDD.
3.13	Acquire excitation lambda stack for red species of KikGR in the green fluorescence channel.
3.14	Turn off green and turn on red NDD.
3.15	Acquire excitation lambda stack for red species of KikGR in the red fluorescence channel.
3.16	Measure the mean pixel fluorescence intensity for each image in the lambda stack. Normalize to the maximum fluorescence.
3.17	Plot the normalized spectral excitation data using Excel or Prism (Figure 5).
Caution	Ensure appropriate IR back filters are in place to allow transmission of shorter excitation wavelengths into the sample. Also make sure the filters prevent reflected laser light from being detected by the NDDs. Be careful not to perform the scan with too high laser power as this will saturate the detectors.
Tip	Set the gain and offset for each detector to optimize the signal-to-noise ratio before performing the fingerprint. These should be the same settings used for a typical experiment. Therefore, we typically do not use frame averaging as this slows down image acquisition. We aim to use lowest laser power possible in our experiments to minimize phototoxicity and photobleaching and therefore typically have our sensitivity gain set toward the high end.

See Fig. 6 for the flowchart of Step 3.

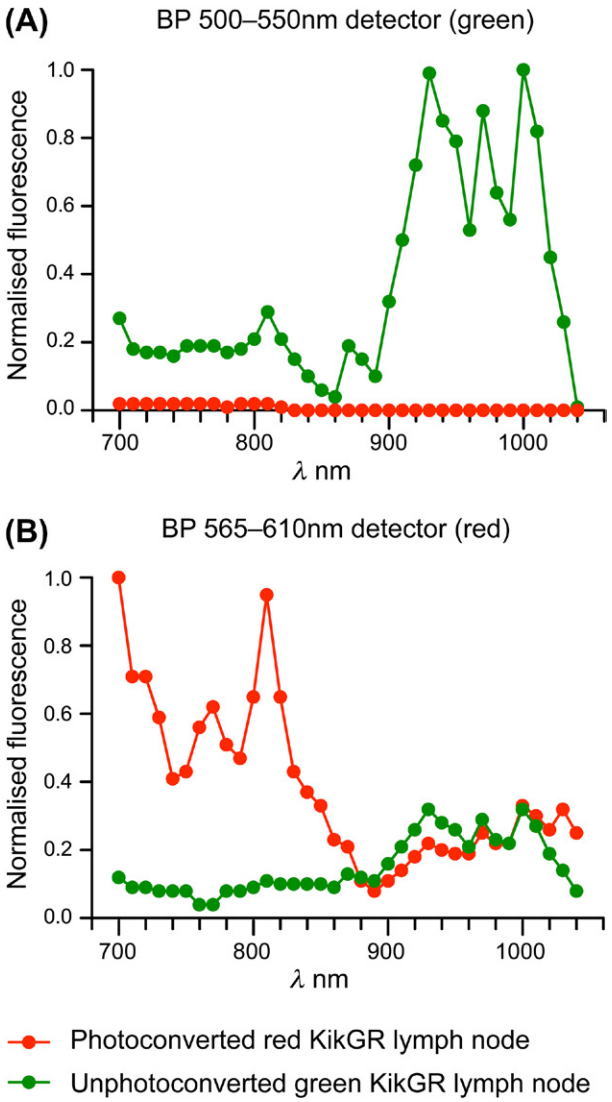

FIGURE 5

Excitation fingerprint of green (green line) and red species (red line) of KikGR. a) Detection of KikGR in the green fluorescence channel. Excitation spectra detected in the green channel shows green KikGR can be excited efficiently between 900 and 1040 nm with multiple excitation peaks at 930, 970 and 1000 nm. In contrast, excitation of red KikGR does not result in any detectable fluorescence in this channel. b) Excitation spectra detected in the red channel shows red KikGR can be excited efficiently at 810 nm. Note the detection of red fluorescence due to cross-talk from green KikGR when excitation wavelengths >900 nm are used. (See color plate.)

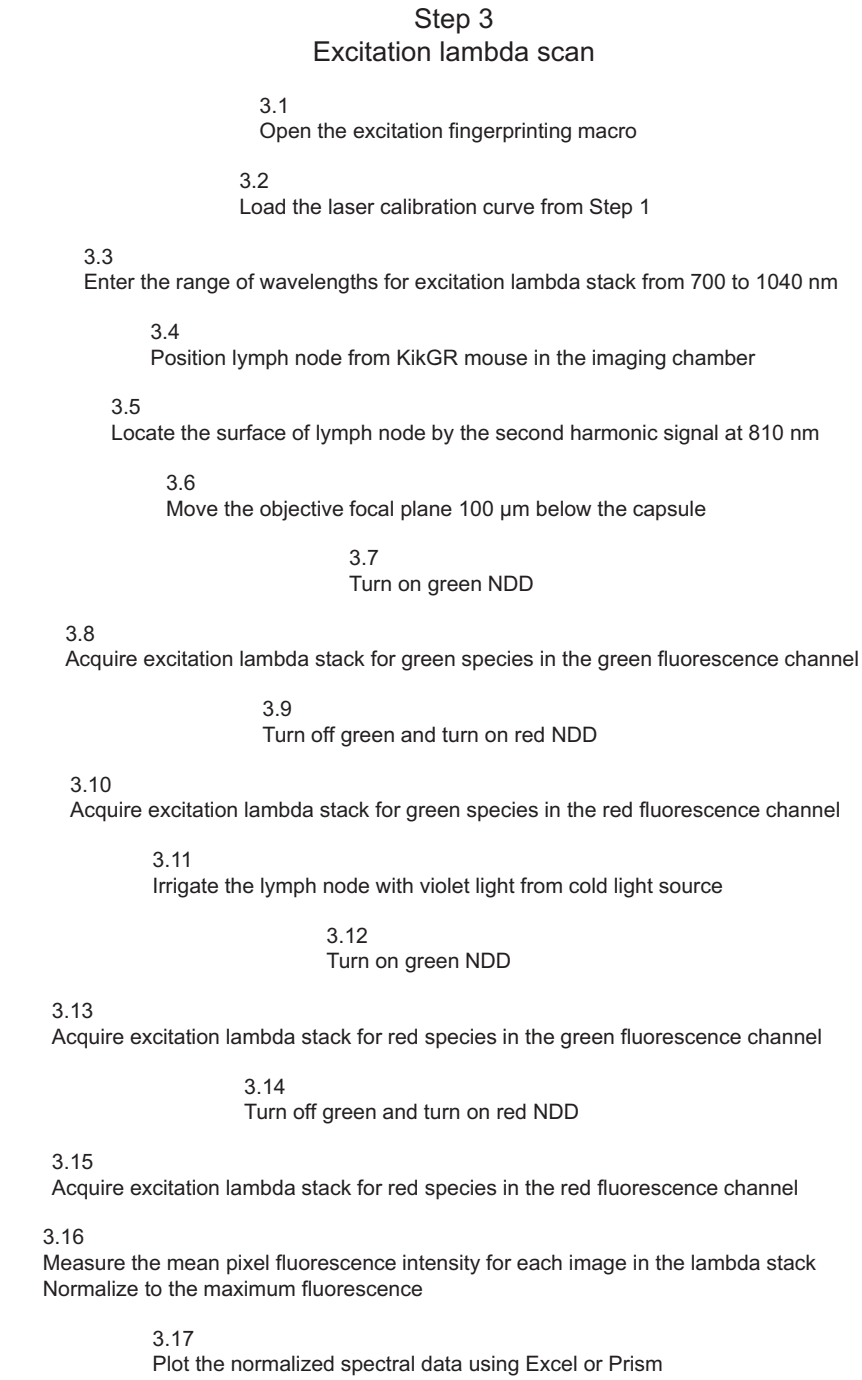

FIGURE 6

Flowchart of Step 3.

Keywords

Keyword Class	Keyword	Rank
Methods List the methods used to carry out this protocol (i.e. for each step).	1 Two-photon microscopy	3
	2 Excitation Lambda scan	1
Process List the biological process(es) addressed in this protocol.	1 Optical highlighter	2
Organisms List the primary organism used in this protocol. List any other applicable organisms.	1 Mouse	4
Pathways List any signaling, regulatory, or metabolic pathways addressed in this protocol.		
Molecule Roles List any cellular or molecular roles addressed in this protocol.		
Molecule Functions List any cellular or molecular functions or activities addressed in this protocol.		
Phenotype List any developmental or functional phenotypes addressed in this protocol (organismal or cellular level).		
Anatomy List any gross anatomical structures, cellular structures, organelles, or macromolecular complexes pertinent to this protocol.	1. Lymph node	5
Diseases List any diseases or disease processes addressed in this protocol.		
Other List any other miscellaneous keywords that describe this protocol.		

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