Journal of Visualized Experiments

Analysis of Lymph Node Volume by Ultra-high-frequency Ultrasound Imaging in the Braf/Pten Genetically Engineered Mouse Model of Melanoma

--Manuscript Draft--

Article Type:	Invited Methods Collection - JoVE Produced Video
Manuscript Number:	JoVE62527R1
Full Title:	Analysis of Lymph Node Volume by Ultra-high-frequency Ultrasound Imaging in the Braf/Pten Genetically Engineered Mouse Model of Melanoma
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TITLE:

Analysis of Lymph Node Volume by Ultra-high-frequency Ultrasound Imaging in the Braf/Pten Genetically Engineered Mouse Model of Melanoma

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KEYWORDS:

Metastatic melanoma, Braf/Pten mice, inguinal lymph nodes, in vivo imaging, ultra-high-frequency ultrasound, 3D rendering

SUMMARY:

Melanoma is a very aggressive disease that quickly spreads to other organs. This protocol describes the application of ultra-high-frequency ultrasound imaging, coupled with 3D rendering, to monitor the volume of the inguinal lymph nodes in the Braf/Pten mouse model of metastatic melanoma.

ABSTRACT:

Tyr::CreER+,Braf$^{CA/+}$*,Pten*$^{lox/lox}$ genetically engineered mice (Braf/Pten mice) are widely used as an *in vivo* model of metastatic melanoma. Once a primary tumor has been induced by tamoxifen treatment, an increase in metastatic burden is observed within 4–6 weeks after induction. This paper shows how Ultra-High-Frequency UltraSound (UHFUS) imaging can be exploited to monitor the increase in metastatic involvement of the inguinal lymph nodes by measuring the increase in their volume.

The UHFUS system is used to scan anesthetized mice with a UHFUS linear probe (22–55 MHz, axial resolution 40 µm). B-mode images from the inguinal lymph nodes (both left and right sides) are acquired in a short-axis view, positioning the animals in dorsal recumbency. Ultrasound records are acquired using a 44 µm step size on a motorized mechanical arm. Afterward, two-dimensional (2D) B-mode acquisitions are imported into the software platform for ultrasound image post-processing, and inguinal lymph nodes are identified and segmented semi-automatically in the acquired cross-sectional 2D images.

Finally, a total reconstruction of the three-dimensional (3D) volume is automatically obtained along with the rendering of the lymph node volume, which is also expressed as an absolute measurement. This non-invasive *in vivo* technique is very well tolerated and allows the scheduling of multiple imaging sessions on the same experimental animal over 2 weeks. It is, therefore, ideal to assess the impact of pharmacological treatment on metastatic disease.

INTRODUCTION:

Melanoma is an aggressive form of skin cancer that often spreads to other skin sites (subcutaneous metastases), as well as to lymph nodes, lungs, liver, brain, and bones[1]. In the last decade, new drugs have been introduced into clinical practice and have contributed to improving the life expectancy of metastatic melanoma patients. However, limitations remain, including variable time to and degree of response, severe side effects, and the insurgence of acquired resistance[1]. Therefore, it is crucial to detect metastatic spreading at its early stages, i.e., when it gets to the local lymph nodes.

A biopsy of the local lymph nodes (sentinel lymph nodes) is usually performed to check for the presence of melanoma cells. However, ultrasound imaging is taking hold as a non-invasive method of detecting metastatic involvement, as it outperforms clinical evaluation and can help avoid an unnecessary biopsy[2-4]. Furthermore, ultrasound imaging seems appropriate for lymph node surveillance, especially in the case of advanced age and/or comorbidities[5,6]. The features that are detected by ultrasound analysis and allow the differentiation between normal and metastatic lymph nodes comprise increased size (volume), change of shape from oval to round, irregular margin, altered echogenic pattern, and altered (increased) vascularization[7].

Tyr::CreER+,Braf$^{CA/+,}$*Pten*$^{lox/lox}$ genetically engineered mice (Braf/Pten mice) have recently been made available to the scientific community as a tissue-specific and inducible model for metastatic melanoma[8]. In this animal model, primary tumors develop very quickly: they become visible within 2–3 weeks after the induction of the switch from wild-type (wt) Braf to BrafV600E and of the loss of Pten, while they reach a volume of 50–100 mm^3 within 4 weeks. In the following 2 weeks, the growth of the primary tumor is accompanied by a progressive increase in metastatic burden in other skin sites, lymph nodes, and lungs.

Braf/Pten mice have been extensively used for multiple purposes including the dissection of signaling pathways involved in melanomagenesis[9,10], the identification of melanoma cells of origin[11-13], and the testing of new therapeutic options~~,~~ in terms of both targeted therapy and immunotherapy[8,14-16]. Specifically, we used Braf/Pten mice to demonstrate that attenuated *Listeria monocytogenes* (Lmat) works as an anti-melanoma vaccine. When systemically administered in the therapeutic setting, Lmat is not associated with overall toxicity~~,~~ as it selectively accumulates at tumor sites and causes a remarkable decrease in primary melanoma mass size and volume and metastatic burden in the lymph nodes and lungs. At the molecular level, Lmat causes apoptotic killing of melanoma cells, which is due, at least in part, to non-cell-autonomous activities (recruitment on-site of CD4+ and CD8+ T lymphocytes)[16].

When Braf/Pten mice are used for melanoma modeling, the growth of primary tumors and subcutaneous metastases can be monitored by caliper measurements. However, the involvement of lymph nodes and lungs needs to be investigated using an alternative technique, possibly a non-invasive one that allows researchers to follow the same animal over time. This paper describes the use of ultrasound imaging (**Figure 1**), coupled with a subsequent 3D volumetric analysis of the obtained data, for the longitudinal monitoring of the increase in size (volume) of inguinal lymph nodes.

PROTOCOL:

All methods described here have been approved by the Italian Ministry of Health (animal protocols #754/2015-PR and #684/2018-PR).

1. Melanoma induction

NOTE: Six-week-old *Tyr::CreER+,Braf*$^{CA/+}$*,Pten*$^{lox/lox}$ mice [*B6.Cg-Braf*^{tm1Mmcm} *Pten*^{tm1Hwu} *Tg(Tyr-cre/ERT2)13Bos/BosJ* (Braf/Pten)] were used in this study (see the **Table of Materials**).

1.1. Treat the mice with 4-hydroxytamoxifen (4-HT) by applying 3 µL of 5 mM 4-HT on ~1 cm^2 of shaved skin of the upper back, as described previously[11,16,17], for 3 days in a row.

NOTE: This will activate the Cre enzyme and cause a switch from wt Braf to BrafV600E and the loss of Pten. These two hits are enough to induce melanoma formation.

1.2. Observe the primary tumors develop at the site of the skin painting in 2–3 weeks and reach a volume of 50–100 mm^3 in 4 weeks. Also, observe metastasis to other skin sites, lymph nodes, and lungs at this time point (t_0).

1.3. Use calipers to measure the volume of the primary tumor and of subcutaneous metastases and ultrasound imaging to measure the volume of the inguinal lymph nodes. Repeat these measurements after one week (t_1, 5 weeks after skin painting) and after two weeks (t_2, 6 weeks after skin painting).

1.4. At the last time point, euthanize the mice by overdosing with gaseous sevorane.

1.5. Analyze the primary tumor and lymph nodes by visual inspection, then excise them for histological studies, as reported in[16].

2. Imaging procedure

2.1. Place the mouse in an induction chamber for gas anesthesia and supply 3% isoflurane in pure oxygen until the animal is fully anesthetized. Verify the depth of anesthesia by lack of response to paw pinch.

2.2. Transfer the animal to a heated board—a constitutive part of the UHFUS imaging station—holding the animal in a supine position. Use a rectal probe lubricated with petroleum jelly to measure the body temperature. Adjust the board temperature to maintain the mouse's body temperature in the physiological range (36 ± 1 °C).

2.3. Moisten the mouse's eyes with vet ointment to prevent dryness during anesthesia. Supply narcotic gas (1.5% isoflurane in pure oxygen) through a mouse's nose mask. Adjust the percentage of isoflurane to maintain the correct depth of anesthesia.

2.4. Coat the fore and hind paws with conductive paste and tape them to the ECG plate electrodes embedded in the board. Check that the physiological parameters (heart rate, respiration signal, and core body temperature) are correctly acquired and displayed.

2.5. Remove hair from both inguinal areas by applying a depilatory agent and coat them with an acoustic coupling medium.

2.6. Clamp the UHFUS linear probe (40 MHz center frequency) into a specialized 3D motor embedded in the UHFUS imaging station, allowing automated and stepwise movement of the probe.

2.7. Properly orient and adjust the position of the ultrasound probe to obtain short-axis images of the inguinal lymph node (left/right), and place the region of interest in the focal zone.

2.8. Scan the entire volume of the inguinal lymph node as a sequence of 2D B-mode images, as described previously[18]. Acquire images at multiple levels of the lymph node by linear movement of the transducer with step sizes on a micrometer scale to generate 3D data in terms of automatically respiration- and cardiac-gated cine loops.

2.9. Set the image recording with the following parameters: scan distance ranging between 2 and 5 mm (depending on lymph node size); step size 44 µm, with an outcome of 46–114 scan steps/lymph node slices and an acquisition time of 1–3 min per animal. Digitally store the acquired images in raw format (DICOM) for further offline analyses.

2.10. At the end of the imaging session, discontinue the gas anesthesia and allow the animal to recover on the heating board in sternal recumbency. Take care of the animal until it has regained sufficient consciousness to maintain the prone position.

3. **Post-processing of ultrasound images**

3.1. Open the dataset of DICOM 3D images of the left/right inguinal lymph node in the software platform for ultrasound image post-processing.

3.2. Segmentation

3.2.1. Select **Multi-sliceMethod** to visualize both the current frames and thumbnails of all frames corresponding to each image captured during the 3D acquisition.

3.2.2. Select the thumbnail of the first frame to load it into the contouring view. In the contouring view, left-click on the mouse to drop points along the border of the lymph node. Once the desired number of points has been set (range 10–15), right-click to complete the contour.

3.2.3. After the first contour is completed, use the thumbnail view to select the next image for contouring. If required, skip over several images (average of 3 frames) between contours to reduce the number of manual traces needed for each 3D volume.

NOTE: The software platform for ultrasound image post-processing will automatically generate contours between manual traces, thereby reducing the time of analysis.

3.2.4. Repeat this process until the entire volume has been outlined. Once complete, click on **Finish**.

3.3. Generation of the 3D wireframe and volume measurement

3.3.1. While in the 3D mode window workspace, click on the **Volume measurement** icon beneath the image display area to activate the **surface view**.

NOTE: The surface view creates a compilation view that maps the user-generated volume to the acquired image. The surface view can be rotated into any desired position.

3.3.2. Take note of the volume measurement listed in the lower left-hand corner of the cube view.

NOTE: Segmentation and 3D volume generation can also be obtained using custom-developed software and/or freely available/commercial software for general image processing. Starting from manual segmentation, the software should provide a mathematical and/or pixel-level description of the lymph node contours. These contours would be combined in a 3D space to render the external surface of lymph nodes. All steps described in the imaging procedure and the post-processing of ultrasound images are summarized in **Figure 2**.

REPRESENTATIVE RESULTS:

After skin painting of *Tyr::CreER+,Braf*$^{CA/+}$*,Pten*$^{lox/lox}$ mice with 4-HT, Cre activity is induced due to which there is a switch at the genomic level from wt *Braf* to BrafV600E, while Pten is lost (**Figure 3A**). In 2–3 weeks, mice develop on-site primary tumors with 100% penetrance. After four weeks from 4-HT treatment (t_0), primary tumors reach a volume of 50–100 mm^3, and their growth can be measured by calipers for an additional 2 weeks ((t_1 and t_2; **Figure 3B**, upper panels). Later time points cannot be reached because the tumor becomes so big that the mice require euthanasia.

As far as the metastatic burden is concerned, a gradual increase in pigmentation is observed in the inguinal lymph nodes within 4–6 weeks from 4-HT treatment (**Figure 3B**, lower panels). Such an increase in pigmentation is due to the presence of melanin deposits, as can be confirmed by hematoxylin and eosin staining performed without removing the melanin. In turn, the melanin deposits are invariably due to the presence of metastasized melanoma cells, as confirmed by immunohistochemical (IHC) staining of the melanoma antigen MLANA and BRAFV600E (**Figure 3C**).

The accumulation of pigmented melanoma cells inside inguinal lymph nodes is accompanied by a progressive increase in their volume, as evident by visual inspection (**Figure 3B**, lower panels). Ultrasound imaging offers the unique opportunity to quantify such an increase longitudinally, in each experimental mouse, as described previously[16]. Volumetric measurements, segmentation results, and 3D rendering, all referring to a representative case, are shown in **Figure 4**. The volume of each lymph node is obtained by manual segmentation of the axial sections acquired during a 3D scan.

At the end of the segmentation phase, all the sections show the overlay of the external contour of the lymph node (**Figure 4A**). These contours are connected frame-by-frame in the rendering phase, and the external surface of the entire lymph node is projected in the 3D space. As a representative example, the 3D rendering of a right inguinal lymph node analyzed at t_0, t_1, and t_2 is shown in **Figure 4B**, **Figure 4C**, and **Figure 4D**, respectively. The graph in **Figure 4E** quantifies the increase in volume displayed by the left and right inguinal lymph nodes of the same animal over time.

FIGURE **AND TABLE** LEGENDS:

Figure 1**: T**he ultrasound imaging system used to monitor the increase in **the** volume of **the** inguinal lymph nodes in the Braf/Pten genetically engineered mouse model of melanoma.

Figure 2: Step-by-step summary of the imaging procedure and the post-processing of ultrasound images.

Figure 3: Visual inspection and histological analyses of inguinal lymph nodes in the tissue-specific and inducible Braf/Pten metastatic melanoma model in the mouse. (**A**) Cre enzyme causes the switch of wt *Braf* into BrafV600E and the loss of Pten (excision of exons 4 and 5). This system is melanocyte-specific because the expression of the Cre enzyme is under the control of the promoter of tyrosinase, an enzyme involved in melanin synthesis. Therefore, the two oncogenic hits are restricted to the melanocytic lineage. This system is also inducible because Cre is expressed as a fusion protein with the estrogen receptor and requires skin painting with 4-HT to be translocated into the nucleus, where it can exert its function. (**B**) The appearance of primary melanoma tumors (upper panels) and inguinal lymph nodes (lower panels) after 4, 5, and 6 weeks after 4-HT treatment (t_0, t_1, and t_2, respectively). In lymph nodes, the increase in melanin accumulation and size is detected by visual inspection. Scale bars = 0.5 cm (upper panels); 0.2 cm (lower panels). (**C**) Histological analyses of inguinal lymph nodes, 6

weeks after 4-HT treatment. H&E staining (upper left): melanin deposits are removed by incubating slices with 1% KOH and 3% H_2O_2. Melanin detection performed by H&E staining without 1% KOH and 3% H_2O_2 treatment (upper right). Bottom left: MLANA detection by immunoperoxidase staining (DAB chromogen substrate and hematoxylin counterstaining). (bottom right) BRAFV600E detection by immunoperoxidase staining (DAB chromogen substrate and hematoxylin counterstaining). For all panels, original magnification: 40x; scale bars = 20 µm. Abbreviations: wt = wild-type; 4-HT = 4-hydroxytamoxifen; H&E = hematoxylin and eosin; DAB = 3,3'-diaminobenzidine.

Figure 4: Measurements, segmentation, and 3D rendering of the volume of the inguinal lymph nodes in the tissue-specific and inducible Braf/Pten metastatic melanoma model in the mouse. (**A**) Overlay of the external contours of the right inguinal lymph node in 4 representative scanned sections obtained by manual segmentation. (**B–D**) Rendering of the 3D volume of the right inguinal lymph node, as measured at 4, 5, and 6 weeks after 4-HT treatment (t_0, t_1, and t_2 time points, respectively). The numerical value of the volume is also reported (in mm^3). (**E**) The volume of the left (black circle) and the right (white circle) inguinal lymph node of the same animal at t_0, t_1, and t_2 time points. Abbreviations: 3D = three-dimensional; 4-HT = 4-hydroxytamoxifen.

DISCUSSION:

The data obtained in this study attest to the ability of ultrasound imaging to monitor the metastatic involvement of inguinal lymph nodes of the Braf/Pten mouse model of metastatic melanoma. As shown previously[16], this technique is especially useful to assess the efficacy of drug treatment. This is because it allows the monitoring of the change in lymph node volume in the same animal over time by comparing the measurements collected at t_1/t_2 with those collected at t_0. This, in turn, contributes to an increase in the robustness of the obtained results, because inter-mouse variability and other factors that might influence basal lymph node size are all accounted for. In addition, ultrasound imaging allows compliance with the 3R principle by reducing the number of animals per experimental group.

In Braf/Pten mice, not only inguinal, but also brachial and axillary lymph nodes are sites of metastatic spreading. However, it is advisable to focus on inguinal lymph nodes because the others are too close to the primary tumor site, which usually alters their localization and morphology during the development of the primary tumor itself. Alternatively, brachial and axillary lymph nodes might become suitable for ultrasound imaging if a different site of tumor induction is chosen, such as ears or paws[8]. As far as other metastatic sites are concerned, lungs cannot be studied using ultrasound imaging, because of the presence of air in the tissue. Theoretically, only superficial pulmonary metastases reaching the pleural interface could be visualized with this technique. Although micro computed tomography/positron emission tomography (CT/PET) could be used instead, this approach has several drawbacks, including high costs and limited availability (being based on ionizing radiation). Furthermore, micro CT/PET is hardly compatible with longitudinal measurements at multiple time points. Conversely, ultrasound imaging can be easily applied to the study of subcutaneous metastases and allows the measurement of both volume and vascularization[16].

If a 2-week time frame is too short to appreciate the effects of the drug under study, a more peripheral induction site (e.g., the tip of the tail[9,11]) or a less tumor-prone genotype (*Tyr::CreER+,Braf$^{CA/+}$,Pten$^{+/lox}$* mice instead of *Tyr::CreER+, Braf$^{CA/+}$, Pten$^{lox/lox}$* mice) could be selected[9]. In both cases, the growth of the primary tumor is expected to be much slower, allowing metastasis monitoring for far more than 6 weeks after induction with 4-HT. From a more technical point of view, it is important to note that 2D segmentation of ultrasound images is the most critical step in this protocol, because it may affect the measurement of 3D volume. Luckily, in the Braf/Pten animal model, the contrast between lymph nodes and surrounding tissues is quite marked, so that the outlining of the lymph node borders by manual segmentation is relatively simple. However, the segmentation process should be facilitated by the high quality of the ultrasound images acquired by the sonographer, who, in turn, should be highly experienced and focused on acquiring the same ultrasound projection of the lymph node, even in scan sessions performed at different time points.

B-mode ultrasound imaging cannot highlight cancer cells directly; instead, it allows the inference of their presence from the increase in the volume of inguinal lymph nodes. In light of this information, it is recommended that ultrasound imaging be coupled with appropriate IHC staining of the lymph nodes, so that the presence of cancer cells can be confirmed at the molecular level. However, a lymph node enlargement observed in an induced Braf/Pten mouse is typically attributable to cancer spreading and not to some other cause , e.g., an ongoing infection. This is likely because experimental mice used for ultrasound imaging are bred in controlled conditions and are routinely subjected to sanitary screening, so that sickness is promptly spotted and treated.

ACKNOWLEDGMENTS:
The authors would like to thank S. Burchielli (FTGM, Pisa) for her assistance with animal procedures. This work was supported by ISPRO-Istituto per lo Studio la Prevenzione e la Rete Oncologica institutional funding to LP; MFAG #17095 awarded by AIRC-Associazione Italiana Ricerca sul Cancro to LP.

DISCLOSURES:
The authors have nothing to disclose.

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Figure 1

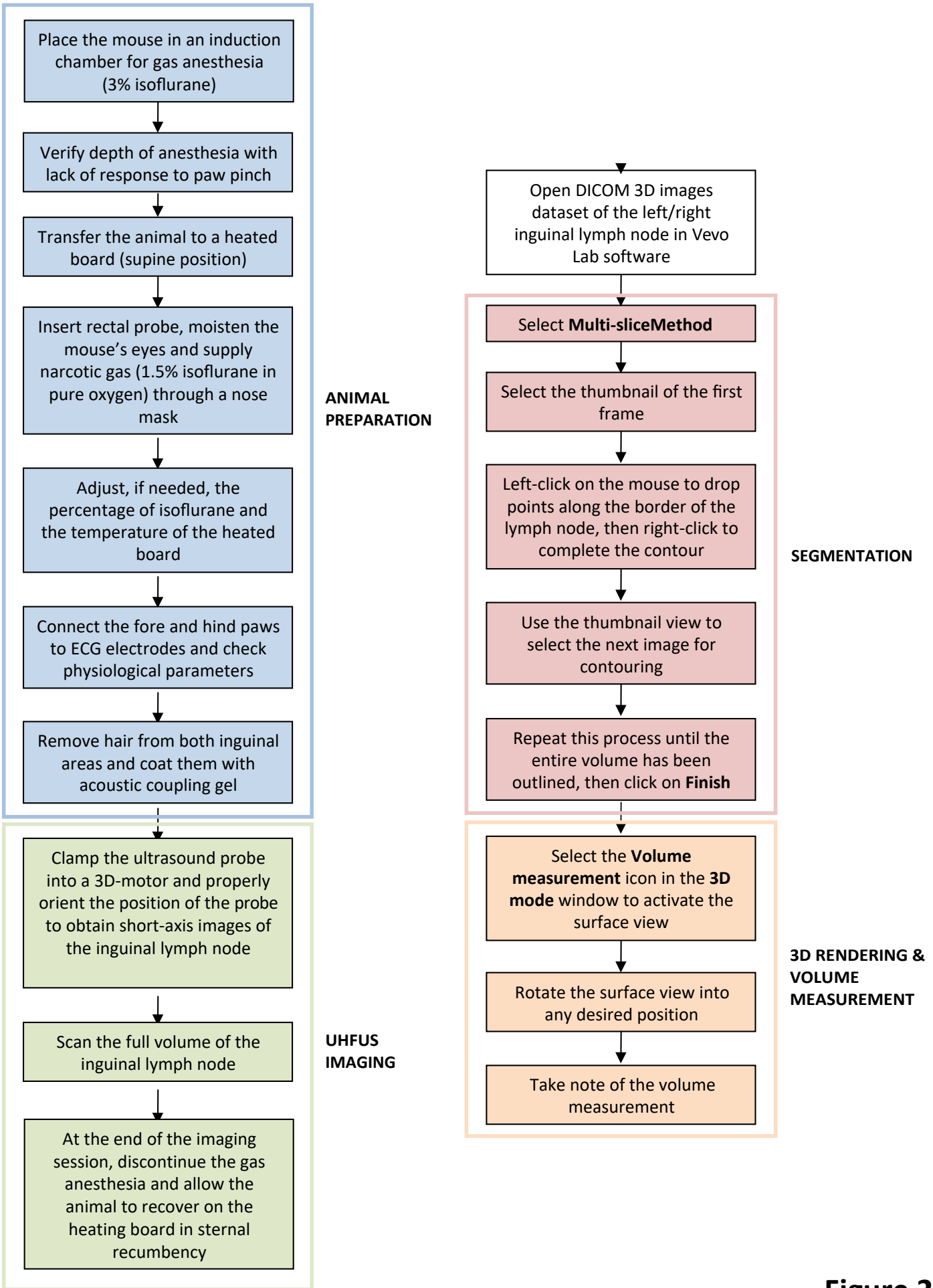

Figure 2

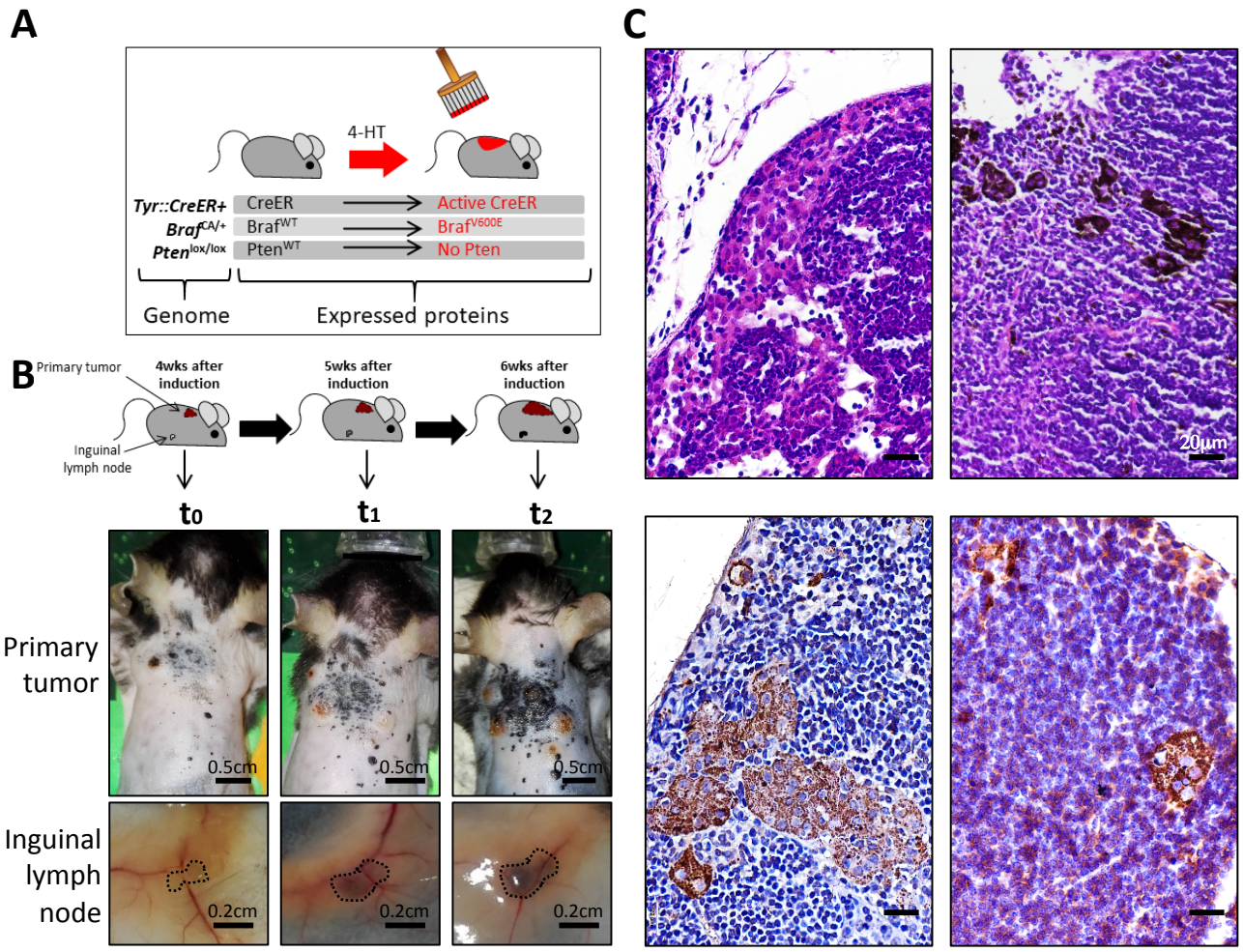

Figure 3

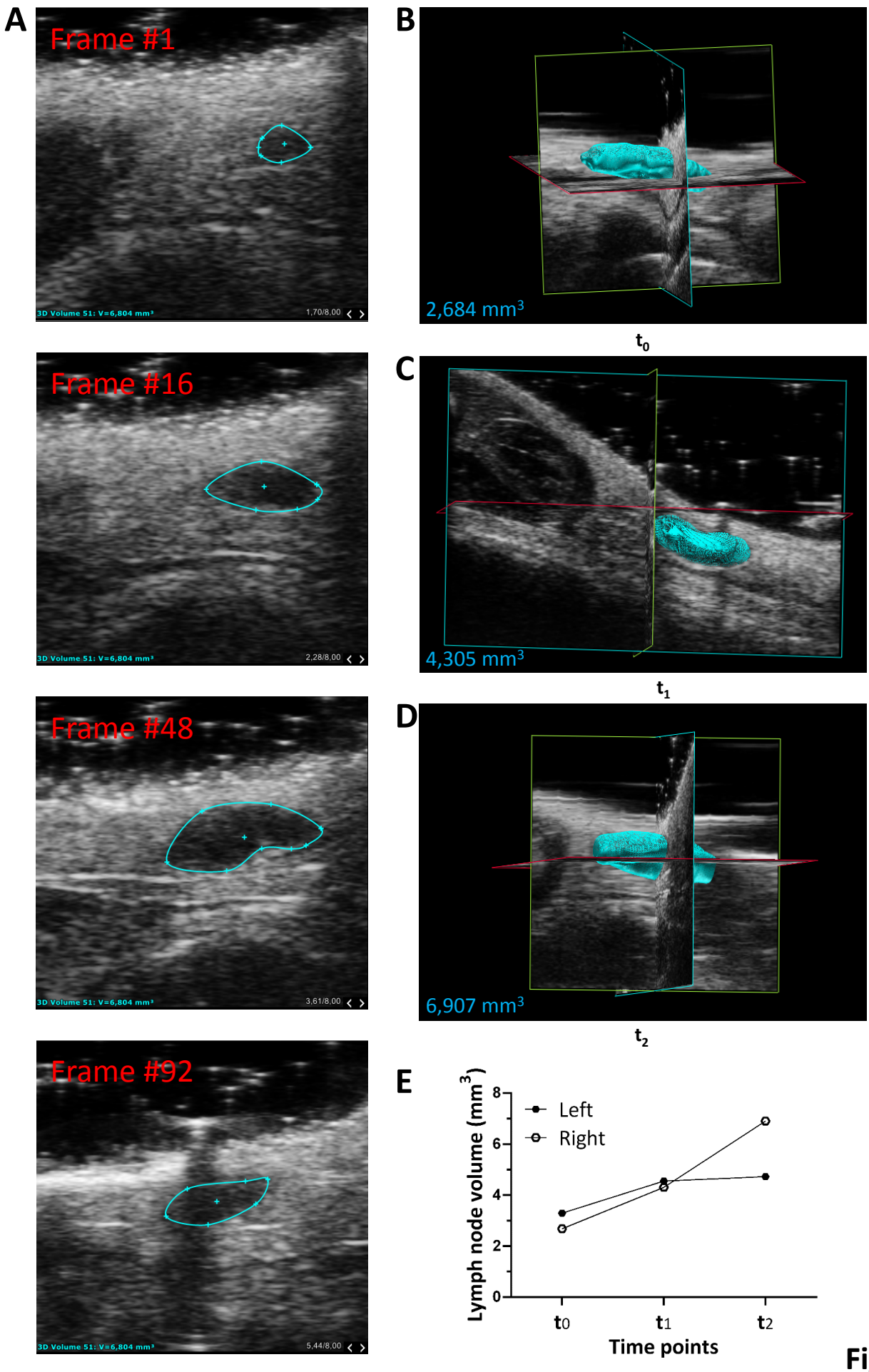

Figure 4

Table of Materials

Name of Material/Equipment	Company	Catalog Number	Comments/Description
4-hydroxytamoxifen	Merck	H6278	drug used for tumor induction
B6.Cg-Braftm1Mmcm Ptentm1Hwu Tg(Tyr-cre/ERT2)13Bos/BosJ (Braf/Pten) mice	The Jackson Laboratory	(#013590)	
Blu gel	Sooft Italia		ophthalmic solution gel
BRAFV600E antibody	Spring Bioscience Corporatio	E19290	
IsoFlo (isoflorane)	Zoetis		liquid for gaseous anaesthesia
MLANA antibody	Thermo Fisher Scientific	M2-7C10	
Sigma gel	Parker		electrode gel
Transonic gel clear	Telic SAU		ultrasound gel
Veet	Reckitt Benckiser IT		depilatory cream
Compact Dual Anesthesia System	Fujifilm, Visualsonics Inc.		Isoflurane-based anesthesia system equipped with nose cone and induction chamber
MX550S	Fujifilm, Visualsonics Inc.		UHFUS linear probe
Vevo 3100	Fujifilm, Visualsonics Inc.		UHFUS system
Vevo Imaging Station	Fujifilm, Visualsonics Inc.		UHFUS imaging station and Advancing Physiological Monitoring Unit endowed with heated board
Vevo Lab	Fujifilm, Visualsonics Inc.		software platform for ultrasound image post-processing

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Pisa, April 12th 2021

Dear Dr. Iyer,
Please find enclosed the revised version of our manuscript entitled "*Analysis of lymph nodes volume by ultra high-frequency ultrasound imaging in the Braf/Pten genetically engineered mouse model of melanoma*-JoVE62527"**.**

We thank you for giving us the opportunity to resubmit and we thank the reviewers for their suggestions, which have certainly contributed to make our experimental protocol more clear and easy to follow. We hope that our manuscript is now considered suitable for publication.

A point-by-point response to the reviewer's concerns can be found below.

Please feel free to contact me, should you need additional information.

Sincerely,

Laura Poliseno, PhD

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Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.
The manuscript has been revised according to the abovementioned instructions.

2. Please revise the following lines to avoid overlap with previously published work: 97-113; 119-121; 123-126; 128-129.
The text was revised, avoiding overlap with previous papers.

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The manuscript has been revised according to the abovementioned instructions.

4. A) Please specify the euthanasia method without highlighting it.
The euthanasia method (overdosing of gaseous sevorane) has been specified, as requested.

B) Please mention how animals are anesthetized and how proper anesthetization is confirmed.
This information was added (see Imaging Procedure step #1).

C) Please specify the use of vet ointment on eyes to prevent dryness while under anesthesia.
This information was added (see Imaging Procedure step #2).

D) For survival strategies, discuss post-surgical treatment of animal, including recovery conditions and treatment for post-surgical pain.
These steps do not apply to our method, which is not a surgery.

E) Discuss maintenance of sterile conditions during survival surgery.
These conditions do not apply to our method, which is not a surgery.

F) Please specify that the animal is not left unattended until it has regained sufficient consciousness to maintain sternal recumbency.
A sentence has been added as point #8 in the Imaging Procedure section.

G) Please specify that the animal that has undergone surgery is not returned to the company of other animals until fully recovered.
This step does not apply to our method, which is not a surgery.

5. Even if you describe how to use this particular software in this paper, please write the protocol to help readers understand (if this is possible) how to adapt the protocol to whatever instrument and software they have available in their labs. This can be done with the help of notes interspersed between the protocol steps.

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A note was added in the Post-processing Imaging section about adapting the protocol to different image processing software packages.

6. Please revise the text, especially in the protocol, to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

The revised text complies with this guideline.

7. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

Protocol section was revised accordingly.

8. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g., button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

We believe that the description of our protocol contains all the abovementioned information.

9. After including a one line space between each protocol step, highlight up to 3 pages of protocol text for inclusion in the protocol section of the video. This will clarify what needs to be filmed.

Protocol text to be included in the video has been highlighted in yellow, as requested.

10. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique.

The Discussion has been revised in order to cover all the points listed above, also taking into account the requests made by all reviewers.

11. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source (italics). Volume (bold) (Issue), FirstPage–LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Please include volume and issue numbers for all references. Please do not abbreviate journal names, and use title case for journal names.

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We have double-checked the references and they are in Jove Endnote style.
12. Please sort the Materials Table alphabetically by the name of the material.
Materials are presented in alphabetical order, as requested.

Reviewer #1:
Manuscript Summary:
The work in this paper focused on volumetric analysis of lymph nodes in mice is interesting and well done.

Major Concerns
I have no major comments.
We thank this reviewer for his/her words of appreciation of our work.

Minor Concerns
line 37: high-frequency ultrasound instead of Ultra High-Frequency UltraSound
We thank the reviewer for this comment. However, we would like to point out that the scientific community largely recognizes and defines the ultrasound technology we work with as Ultra High-Frequency Ultrasound (UHFUS) imaging [1 2 3]. In particular, such technology is characterized by the use of probes with frequency ranging from 22 MHz up to 100 MHz. It is therefore different from clinical ultrasound systems that use high-frequency ultrasound probes with frequency in the 10-22 MHz range.
line 38: replace to US imaging.
Please refer to the above answer about UHFUS definition.
line 39: replace to Vevo 3100 imaging modality (FUJIFILM VisualSonics, Inc.).
All definitions of devices and softwares have been revised as for Editorial recommendations and they are summarized in the Table of materials.
line 42: Ultrasound records are acquired using an 44 µm step size on a motorized mechanical arm.
Text was revised as suggested.
line 60: replace to US-imaging.
Please refer to the above answer about UHFUS definition.
line 76: coupled with a subsequent 3D volumetric analysis of obtained data.
Text was revised as suggested.
line 115: replace to Clamp the Mx550S US probe (40 MHz center frequency) into...
Text was revised as suggested.
line 132: Vevo LAB software.
Text was revised as suggested.

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Reviewer #2:

The manuscript presents the analysis of lymph nodes volume by ultra-high frequency US imaging of the mouse melanoma tumor. The authors have presented the methods of growing tumor in the skin of the mouse and the observations results are presented. They have tried to monitor the volume of the inguinal nodes in the Braf/Pten model of metastatic melanoma in the mouse. There is some concern that needs to be addressed:

Abstract

1. The abstract is more descriptive. It does not give the reader about the whole idea of this research as well as the findings of the research. Try to start with some background study followed by the problem statement. Then present the method and the findings onward.

The abstract has been revised taking into account the reviewer's suggestions.

Introduction

2. The authors should add some literature in this section. They have put the method of how to generate the tumor in the rat skin. The best way is to put the method paragraph inside the "Melanoma Induction" section.

Following the reviewer's suggestions, we have added more literature in the Introduction and we have moved the induction procedure to the Protocol section.

3. In the last paragraph of the introduction, talk more about your research findings.

The last paragraph of the introduction has been expanded as requested.

Methodology

4. The procedures of the imaging described in detail. However, its recommended to make a flow diagram or a pictorial representation of the steps.

A flow diagram of the imaging procedure has been added as requested (see new fig.2).

5. In Post Processing section, the authors should make a flow diagram or a visual representation.

A flow diagram of the post-processing procedure has been added as requested (see new fig.2).

6. In imaging section, the authors state that the step size is 44µm. Is it possible to get even smaller steps for better resolution?

The minimum step size for Vevo 3100 is 38 µm. However, the choice of 44 µm step size is a perfect compromise for both optimal 3D reconstruction and acquisition time.

7. What are the frame rates for the scanning?

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The concept of frame rate does not apply to our method, as we collect 1 frame for each step of the 3D volume. See also our answer to point #6 above.

8. Authors should add an image of the overall screening system.

A picture of the screening system has been added as requested (see new fig.1).

9. Why is the temperature fixed at 40 °C?

The temperature of the heated board was set to 40°C in order to keep the body temperature of mice (measured by rectal probe) in a physiological range (36 ± 1°C). In order to ease reproducibility, the description of the procedure aimed at maintaining the mice body temperature was revised adding more details.

Results and Discussions

10. The results section is not arranged, its difficult to track what are the things are presented.

Results have been rearranged and we hope that now are easier to read.

11. The presentation of the image should be clearer. Authors can split the images rather than putting in single legends.

In order to increase clarity and favor the implementation of the method we describe, we have added 2 figures (one showing the ultrasound imaging system that we use for our experiments (new fig.1) and another one summarizing the steps of the imaging procedure and of the post-processing of ultrasound images (new fig.2). In addition, we have simplified and increased the visibility of ex fig.2-revised fig.4 (please see point #12 below). As far as ex fig.1-revised fig.3 is concerned, we have introduced the changes requested by all reviewers, but we prefer not to split it, as it is crucial for us to make the point that there is a correspondence between the increase in lymph node size/melanin accumulation, which can be appreciated by visual inspection (panel B), and the presence of melanoma cells, which can be confirmed using appropriate IHC stainings (panel C).

12. Figure 2(A) should be separated into several image for increasing the visibility.

In order to increase visibility, revised fig.4A now contains fewer, hence bigger panels, which give an overview of the contours of the lymph node across the scanning procedure.

13. Authors did not conclude about the findings. The observations could be extended in few more weeks.

We thank the reviewer for this comment. However, as we mention in the text, mice require euthanasia within 6 weeks after induction, due to the size of their primary tumor. Therefore, it is not possible to extend observations any longer. We also would like to point out that, in compliance with the scope of JoVE articles, we have focused on the technical aspects of the protocol we describe, while we do not show new experimental results that need to be discussed.

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Reviewer #3:

Manuscript Summary

The authors describe an ultrasound based method how to measure the volume of inguinal lymph nodes in the Braf/Pten model of metastatic melanoma in the mouse over time (6weeks).

General comments

I find the manuscript interesting and I think it is good to highlight a non-invasive technique to monitor animals over time. In general I think the manuscript is written in a way which makes it possible to repeat the measurements by following the manuscript.

We thank this reviewer for his/her words of appreciation of our work.

What I miss the most is a comparison of how this presented ultrasound based segmentation technique can measure the real volume of the lymph nodes. Is the volume calculated by the VevoLab software similar to the actual volume of the lymph nodes?

Unfortunately, the comparison between *in vivo* and *ex vivo* lymph nodes volume measurements is not available to us. However, we would like to point out that the VevoLab software is supposed to produce volume measurements that are very close to reality, once an accurate segmentation of 2D sections is provided. We also would like to point out that our aim is to measure changes in lymph node volume across different time points, rather than absolute values. Accordingly, real volume values are not of crucial importance for the implementation of our methodology.

And how user independent is this technique, is it easy to distinguish lymph node tissue from the other tissue in the ultrasound images?

In general, the greater is the difference in acoustic impedance between two tissues, the more reliable is the ultrasound segmentation that can be obtained. In the case of the melanoma model we use (Braf/Pten mice), the difference in acoustic impedance between lymph nodes and surrounding tissues is quite large, thus allowing a fast and reliable manual segmentation. On the other hand, a reliable segmentation strongly depends on the quality of acquired images. In that sense, in our experiments all images were acquired by the same experienced sonographer, who paid particular attention on acquiring the same ultrasound projection of the lymph node, even in scan obtained at different time points.

Now the manuscript describes the method, but there is no evaluation how well the technique perform. It is also a bit unclear if the authors are following Visualsonics guidelines for how to use the VevoLab software or if they have developed something on their own. At row 49 they write: "Our non-invasive in vivo technique is very well tolerated and multiple imaging sessions can be scheduled on the same experimental animal" but to me it seems to be Visualsonics technique which the authors are using in a nice and appropriate way.

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We agree with the reviewer. “Our non-invasive technique ..” was changed into “This non-invasive technique ..”, as the paper describes a specific application of the VevoLab software for 3D volume measurement.

Specific comments
Introduction
Line 56: which kind of therapeutic challenges?
The first paragraph of the introduction has been rephrased and clarified, according to the reviewer’s request.
Line 58: The LN abbreviation is only used here in the running text. It is also used in the figure legend to fig 1 (three times) and in fig 1b. I think it would be better to skip the LN abbreviation and write lymph node instead at these 4 sites in the manuscript.
The abbreviation “LN” has been removed from the manuscript, as requested.
Line 60: I think it would be interesting if authors clarify a little more how the ultrasound was used in this study.
Following the reviewer’s suggestion, we have better described the clinical use of ultrasound.
Line 76-77: I think that "ultra high-frequency" should be moved in this sentence since it is the ultrasound that is high frequency and not the reconstruction.
"In this work we show how to exploit ultrasound imaging, coupled with 3D ultra high-frequency ultrasound reconstruction" be changed to "In this work we show how to exploit ultra high-frequency ultrasound imaging, coupled with 3D ultrasound reconstruction".
The text was changed accordingly (please also refer to the request of Reviewer #1).
Melanoma induction
Line 90: How do you measure the volume with the caliper? Measure the length and width, assume an elliptic structure and calculate the volume or do you do something else?
Indeed, we use the modified ellipsoidal formula $V=(width^2 \times length)/2$.

Imaging procedure
Line 101: Is the temperature-controlled board something which is a part of the Vevo 3100 equipment? Maybe a part of the Vevo Imaging Station mentioned at line 116? I think this board should be mentioned in the equipment table.
Following the reviewer’s suggestion, the text was changed, specifying that the heated board is a component of the Vevo Imaging Station. This is now mentioned in the Table of materials as well.
Line 103: Why is 2,5% isoflurane used in the chamber and 1,5% in the nose cone?
We use a higher percentage of isoflurane in the induction chamber to allow a rapid stunning of the mouse, thus reducing animal stress. During the imaging session, a lower percentage of

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isoflurane is sufficient to maintain the sedation, without depressing the physiological parameters (respiratory and heart rate) outside acceptable ranges. In fact, any type of anesthesia (gaseous or chemical), by interacting with the central nervous system, tends to depress physiological parameters and it is therefore important to find an acceptable compromise.

Line 104: How do you control if the percentage isoflurane is enough?

We check the absence of paw reflex, in the presence of respiratory and heart rate values of 120± 20bpm and >400bpm, respectively.

Line 105: Isn't 40 degrees C a bit warm? 36.6 degrees C seems to the body temperature of a mouse.

Please refer to the answer below.

Line 108: Do you use this initial temperature measurement as the temperature the animal should have during the measurement and then you adjust the temperature with temperature controlled board? This procedure could be explained clearer I think.

In order to address the issues raised also by other reviewers, we added a more detailed description of the procedure aimed at maintaining mice body temperature. We monitor mice body temperature by a rectal probe and keep the temperature of the heated board at 40°C. In this way, we ensure that the animal is maintained in its physiological range of body temperature (36 ± 1°C).

Line 115: US is not explained before and is only used in point 5 and 6 in the imaging procedure. I think it is better to write ultrasound probe instead of US probe at line 115 and 119.

Text was revised as suggested.

Line 116: I think the Vevo Imaging Station should be mentioned in the equipment table.

All definitions of devices and software have been revised as for Editorial recommendations and are listed in the Table of materials.

Line 119-121: Why do you scan like this, is it the easiest way, the way which gives you most cross sections or something else?

Short axis views are chosen because they are the optimal view for manual segmentation and subsequent 3D reconstruction, due to the “rice shape” of lymph nodes. However, in line of principle, also alternative view (e.g. long axis view) could be used for 3D volume measurement.

Line 123: Perhaps add B-mode to 2D images -> 2D B-mode images.

Text was revised as suggested.

Line 156: 3.3 is set as bold text, change to normal text

This change has been introduced, as requested.

Representative results

Line 166: What is the size of these primary tumors after 6 weeks?

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After 6 weeks from induction, control mice (no pharmacological treatment) show primary tumors that are ~3cm^3.

Line 167-168: "As far as inguinal lymph nodes are concerned, within 4-6 weeks from 4-HT treatment they invariably undergo a gradual increase in their melanin content". If the melanin content increases in a lymph node, is this another way to say that the cancer has spread to this lymph node? Is the melanin always increasing in the sites in the body to where the melanoma cancers spreads? Is it always possible to see this with the naked eye when the lymph node is exposed (e.g. during surgery)?

In our experience with the Braf/Pten model of metastatic melanoma, when a lymph node looks “dark” at visual inspection, this invariably means that it has been reached by pigmented melanoma cells, as can be confirmed by IHC staining of the melanoma antigen MLANA or of BRAFV600E (see also below) and is reported in refs. [4] [5]. Conversely, we observed that metastatic nodules to the lung can be non-pigmented [6]. So, it appears that the site of metastasis has an impact on pigmentation status of melanoma cells [7].

Line 168-171: Why was the histology performed? To confirm that cancer cells were present in the lymph nodes? Please clarify this.

According to the reviewer’s suggestion, in the revised text we have clarified that IHC was indeed performed to confirm that cancer cells are present in the lymph nodes.

Is it possible to see in the ultrasound images if cancer is present in the lymph node or is it just the lymph node size change which is registered with the ultrasound?

Unfortunately, ultrasound imaging only provides morphological images, as it is based on differences in the acoustic impedance of tissues. On the other hand, the rapid increase in volume of lymph nodes has been widely recognized as a reliable marker of the presence of cancer cells [8].

Line 177: On row 177 your write "longitudinal sections acquired during 3D scan" and on line 119-121 "obtain short axis images of the (left/right) inguinal lymph node". Is longitudinal sections and short axis images the same thing or am I misunderstanding this.

Thank you for the observation. All images were acquired from axial section. Text was revised as suggested.

Line 182-184: Is the results in 2E also similar to reality, i.e. when the lymph node volume is investigated outside the body with some additional technique e.g. histology or whatever could be suitable. As I wrote as a general comment it is hard to know well this ultrasound calculated volume is compared to the real size and thus it is hard to know how well the technique works.

Please refer to our answer to the general comment.

Fig 1b: Inguinal LN, It would be nice to have a scale bar in this image if possible and preferably write lymph node instead of LN since LN is nearly never used.

The panel has been modified as requested.

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Fig 1c (line 201): 20µm could be written by the scalebar in the image so you can get a feeling of the size by just looking at the image instead of digging in to the figure legend text.
The panel has been modified as requested.

Discussion
Line 238: Do you have a suggestion what technique to use to measure metastases in the lungs?
microCT/PET could be used to visualize lung metastases in mice models. However, this approach has several drawbacks, including high costs and limited availability (being based on ionizing radiation). Furthermore, it is not compatible with longitudinal measurements at multiple time points.
It would be good if you could discuss the difficulty of outlining the lymph node boarders in ultrasound images and how that may affect the resulting volume and thus the results. Is it easy to differentiate lymph node tissue from the rest of the tissue in the ultrasound images?
In our model, the contrast between lymph nodes and surrounding tissues is quite marked, thus allowing a relatively simple outlining of the lymph nodes borders by manual segmentation. Accordingly, the operator who performed such analysis required minimal training. However, segmentation was facilitated by the high quality of ultrasound images acquired by the sonographer, who is highly experienced and focused on acquiring the same ultrasound projection of the lymph node, even in scan obtained at different time points.
I don't know if this is relevant in mice but in humans (as far as I understand) lymph nodes can be enlarged when the immune system is working with an ongoing infection. Is it possible to distinguish between these kinds of enlargements and enlargements due to cancer spread to the lymph node?
We agree with this reviewer that both infection and cancer can spread to lymph nodes and cause their enlargement, while ultrasound imaging measures volumes and changes in volume, irrespectively of the cause that produced them. Nevertheless, it has never occurred to us that lymph node enlargement in a Braf/Pten induced mouse was due to other than melanoma spreading, likely because experimental mice are bred in controlled conditions in a state-of-the-art animal facility and are routinely subjected to sanitary screening, so that infections are promptly identified and treated.
Can you get some information about cancer infiltration in the ultrasound images or is it just the size which can be measured?
To date, as we describe in the manuscript, we have used ultrasound imaging to measure the increase in metastatic spreading, taking the increase in the volume of inguinal lymph nodes as read out. Currently, our experimental efforts are aimed at assessing whether the presence of melanoma cells causes a change in the ultrasound texture of inguinal lymph nodes as well.

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