Consiglio Nazionale delle Ricerche
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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.

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript (abstract, main text, figure legends; the only place brand names and sources can appear is in the Table of Materials; use generic terms instead in the manuscript. Enter the generic description used in the text in the comments column of the Table of Materials. For example: Vevo 3100 UHFUS system (FUJIFILM VisualSonics Inc., Toronto, Canada); MX550S linear probe etc.

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.

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.

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?

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.

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

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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2   Puma, A. *et al.* Comparison of high-frequency and ultrahigh-frequency probes in chronic inflammatory demyelinating polyneuropathy. *Journal of Neurology.* **266** (9), 2277-2285, (2019).

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6   Vitiello, M. *et al.* Antitumoral effects of attenuated Listeria monocytogenes in a genetically engineered mouse model of melanoma. *Oncogene.* 10.1038/s41388-019-0681-1, (2019).

7   Kim, I. S. *et al.* Microenvironment-derived factors driving metastatic plasticity in melanoma. *Nat Commun.* **8** 14343, (2017).

8   Jayapal, N. *et al.* Differentiation Between Benign and Metastatic Cervical Lymph Nodes Using Ultrasound. *Journal of Pharmacy and Bioallied Sciences.* **11** S338-S346, (2019).