Journal of Visualized Experiments

Longitudinal intravital imaging of brain tumor cell behavior in response to invasive surgical biopsy

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE59278R1
Full Title:	Longitudinal intravital imaging of brain tumor cell behavior in response to invasive surgical biopsy
Keywords:	Intravital imaging, Glioma, Biopsy, Migration, Proliferation, Cranial imaging window
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Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Open Access (US$4,200)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Utrecht, the Netherlands

To: Dr Bajaj

Science Editor

Dear Dr. Bajaj,

We would like to thank the reviewers for their time reviewing our manuscript, “Longitudinal intravital imaging of brain tumor cell behavior in response to invasive surgical biopsy”. We are very pleased with the overall positive evaluation of our manuscript and appreciate the constructive comments, which we have addressed in the attached point-by-point rebuttal. We hope these changes make this manuscript now fully suited for publication in JOVE.

Best wishes,

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TITLE:

Longitudinal Intravital Imaging of Brain Tumor Cell Behavior in Response to an Invasive Surgical Biopsy

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KEYWORDS:

Intravital imaging, glioma, biopsy, migration, proliferation, cranial imaging window

SUMMARY:

Here we describe a method for high-resolution time-lapse multiphoton imaging of brain tumor cells before and after invasive surgical intervention (e.g., biopsy) within the same living animal. This method allows studying the impact of these invasive surgical procedures on tumor cells' migratory, invasive, and proliferative behavior at a single cell level.

ABSTRACT:

Biopsies are standard of care for cancer treatment and are clinically beneficial as they allow solid tumor diagnosis, prognosis, and personalized treatment determination. However, perturbation of the tumor architecture by biopsy and other invasive procedures has been associated with undesired effects on tumor progression, which need to be studied in depth to further improve the clinical benefit of these procedures. Conventional static approaches, which only provide a snapshot of the tumor, are limited in their ability to reveal the impact of biopsy on tumor cell behavior such as migration, a process closely related to tumor malignancy. In particular, tumor cell migration is the key in highly aggressive brain tumors, where local tumor dissemination makes total tumor resection virtually impossible. The development of multiphoton imaging and chronic imaging windows allows scientists to study this dynamic process in living animals over time. Here, we describe a method for the high-resolution longitudinal imaging of brain tumor cells before and after a biopsy in the same living animal. This approach makes it possible to study the impact of this procedure on tumor cell behavior (migration, invasion, and proliferation). Furthermore, we discuss the advantages and limitations of this technique, as well as the ability of this methodology to study changes in the cancer cell behavior for other surgical interventions, including tumor resection or the implantation of chemotherapy wafers.

INTRODUCTION:

Standard of care for most solid tumors includes tissue biopsy for diagnosis, prognosis, and personalized treatment determination[1,2]. Overall, these procedures give clinical benefit, but recent evidence indicates that biopsy and other more invasive procedures, such as tumor resection, can also negatively influence tumor progression[3–6]. While these procedures remain indispensable in-patient care and their benefits overcome their negative effects, it is necessary to fully understand the mechanisms behind these negative effects in order to maximize the patients' safety and the positive influences of these procedures and make them even more clinically beneficial.

Biopsy-mediated undesired effects on tumor progression are triggered by systemic alterations and changes in the tumor microenvironment in response to tissue disruption[4,5]. Thus, it is necessary to study this process in live animals. However, the subtle consequences of these minimally invasive procedures can often be disguised by large variations between individuals. Conventional methods based on immunohistochemistry or transcriptional expression analysis may overlook these effects or require large numbers of animals to identify them. Moreover, these static approaches lack the ability to identify changes in tumor cell behavior such as migration and invasion, dynamic processes that correlate with tumor malignancy. These tumor cell features are of particular importance for highly aggressive brain tumors, such as glioblastoma multiforme (GBM), where the local spreading of tumor cells limits surgical resection and decreases patient survival[7]. To fully understand how biopsies affect the behavior of GBM cells, a longitudinal approach that allows visualization of these cells in the physiological context of living organisms is needed.

The recent development of high-resolution intravital imaging in combination with surgically implanted chronic imaging windows allows scientists to study the dynamic behavior of tumor cells in living mice over multiple days[8,9]. Using this powerful approach, we can study how tumor cells' proliferative, migratory, and infiltrative behavior changes over several days in response to a biopsy in the same mouse. Compared to other techniques that allow multi-day monitoring of tumors in live mice, such as magnetic resonance imaging (MRI)[10], positron emission tomography/computed tomography (PET/CT)[11], or bioluminescent imaging[12], this approach uniquely offers the possibility of studying the tumor cell behavior at single cell level and unraveling subtle changes occurring within the tumor.

Here, we describe a detailed method to perform biopsy-like injury and pre- and postbiopsy longitudinal intravital imaging in the brain of tumor-bearing mice. This method can potentially be applied to study other surgical interventions, such as partial tumor resection or the implantation of chemotherapy wafers.

PROTOCOL:

All experiments were carried out in accordance with the guidelines of the Animal Welfare Committee of the Royal Netherlands Academy of Arts and Sciences, the Netherlands. The experimental protocols used in this manuscript were approved by the Centrale Commissie Dierproeven (CCD) and the Instantie voor Dierenwelzijn (IvD).

1. Tumor cell implantation and cranial imaging window preparation

1.1. Surgical preparation

1.1.1. Use adult mice (>6 weeks old) of any strain or gender. Sedate a mouse by injecting (fluanisone [neuroleptic] + fentanyl [opioid]) (0.4 mL/kg) + benzodiazepine sedative (2 mg/kg) at a dose of 1:1:2 in sterile water. Assess the animal's sedation state by toe pinch.

NOTE: In this protocol, both female and male C57BL/6 mice were used due to the same genetic background of the tumor cell line used in these experiments (GL261). The mouse will stay fully sedated for 1.5 h. Alternatively, use inhalation anesthesia such as isoflurane (1.5%–2% isoflurane/O_2 mixture).

1.1.2. Mount the mouse on a stereotactic frame and secure the head using a nose clamp and two ear bars.

1.1.3. Use a heating lamp to preserve body temperature.

1.1.4. Apply eye ointment to protect the mouse's corneas from drying.

1.1.5. Use sharp scissors to shave the fur on the skull (dorsal area from the mouse's eyes to the base of its skull) and sterilize the exposed skin with 70% ethanol.

1.1.6. Cut the skin in a circular manner with sharp scissors and scrape away the periosteum underneath with a cotton swab. Apply a drop of lidocaine 1% + epinephrine 1:100,000 for 5 min, and remove the excess with a cotton swab.

1.1.7. Glue the edges of the skin to the skull with cyanoacrylate glue.

1.1.8. Place the stereotactic frame under a dissection stereo microscope with 4x magnification.

1.1.9. Visualize the skull through the dissection microscope and drill a circular groove of 5 mm in diameter over the right parietal bone. Perform this step carefully and only superficially, avoiding any pressure on the skull.

1.1.10. Apply a drop of cortex buffer (125 mM NaCl, 5 mM KCl, 10 mM glucose, 10 mM HEPES buffer, 2 mM $MgSO_4$, and 2 mM $CaCl_2$ [pH 7.4]) and lift the bone flap using thin forceps.

1.1.11. For the following steps, keep the brain surface covered with cortex buffer unless indicated otherwise.

1.1.12. Under the dissection microscope, visualize the brain surface and remove the dura mater using curved, tapered, very fine point tweezers. If bleeding occurs at this stage, use an absorbable gelatin sponge to stop it.

1.2. Tumor cell injection

1.2.1. Resuspend the desired amount of fluorescent tumor cells in ~3 µL of PBS (e.g., for the experiments shown in this protocol, 1 x 10^5 GL261 cells were injected).

NOTE: While any fluorescent marker can be used, a nuclear marker is strongly advised, to track individual tumor cells during analysis. Additionally, a histone-linked fluorescent marker, such as H2B, can be used to visualize chromosome condensation and, thus, to monitor cell division. The use of a photo-switchable marker, such as Dendra2, allows the photo-marking and tracking of tumor cells over several days[4,13].

1.2.2. Load the tumor cells in a 10 µL gas-tight syringe with a point style 2 needle and fix it on the stereotaxic manipulator arm.

1.2.3. Remove the cortex buffer. Place the tip of the syringe in the middle of the craniotomy and insert it at a depth of 0.5 mm from the surface of the skull. Optionally, create a small space in the brain to accommodate the tumor cell suspension. For this, insert the syringe up to a depth of 1 mm and then retrieve it up to 0.5 mm before the injection.

1.2.4. Apply a drop of cortex buffer.

1.2.5. Slowly inject the cell suspension using a microsyringe pump injector (250–400 nL/min). Remove the syringe and use an absorbable gelatin sponge to stop any bleeding, if necessary.

1.3. Cranial imaging window preparation

1.3.1. Remove the cortex buffer and place a drop of silicone oil on the craniotomy site to avoid air bubbles under the window.

1.3.2. Seal the exposed brain with a 6 mm coverslip. Apply cyanoacrylate glue between the coverslip and the skull. Gently press the coverslip against the skull with the help of fine tweezers to ensure minimal distance between the brain and the coverslip.

1.3.3. Apply dental acrylic cement on the skull's surface, covering the edge of the coverslip. Place a thin stainless-steel ring (1.5 mm in outer diameter, 1 mm in inner diameter) around the craniotomy and allow the dental cement to dry. Optionally, add some glue on the border to secure the ring on the top of the head.

NOTE: This head fixation system is optimal for imaging on an inverted microscope: small magnets embedded in the imaging box facilitate the cranial imaging window (CIW) fixation. In the experiments shown in this protocol, an inverted microscope was used. For upright microscopes, fixation can be achieved by means of a bar or a ring with grooves that can be attached to the microscope with the help of screws or a plate that fits the ring.

1.3.4. For pain management, inject a single dose of 100 µg/kg buprenorphine subcutaneously and allow the animal to recover on a heating pad.

1.3.5. Place the mouse in an individual cage with shredded paper for enrichment. Closely monitor the mouse daily for the first few days and 2x per week, thereafter, for normal behavior, reactivity, and appearance.

2. Intravital imaging

NOTE: The time interval between the tumor cell injection and the first intravital imaging session is dependent on the type of tumor cell line used. For the experiments shown in this protocol, 1x 10^5 GL261 cells were injected and imaged 10 days later.

2.1. Imaging preparation

2.1.1. Sedate the mouse using isoflurane inhalation anesthesia through a face mask (1.5%–2% isoflurane/O_2 mixture).

2.1.2. Inject the mouse subcutaneously with 100 µL of saline buffer to prevent dehydration.

NOTE: For long-term imaging, the mouse can be hydrated through a subcutaneous infusion pump.

2.1.3. Place the mouse face-up in an imaging box. Use a metal plate with a 1 mm-diameter hole and small magnets embedded around the aperture to provide fixation of the CIW to the imaging box. Introduce isoflurane through a facemask and ventilate by an outlet on the other side of the box (0.8%–1.5% isoflurane/O_2 mixture). Optionally, use tape to fix the body of the mouse.

NOTE: Fluorescently labeled dextran for blood vessel visualization or other dyes may be injected intravenously at this point.

2.1.4. Optionally, use a pulse oximeter and a heating probe to monitor the mouse's vitals.

NOTE: In this experiment, it was not necessary to use a pulse oximeter and a heating probe since the mouse was stable throughout the imaging time period (2–3 h). However, for longer imaging times, more thorough monitoring may be needed.

2.1.5. Set the 25x (e.g., HCX IRAPO NA0.95 WD 2.5 mm) water objective to the lowest *z*-position and add a large drop of water.

NOTE: The use of a water immersion micro dispenser is highly advised for long term experiments since it allows scientists to add water during the experiment. Alternatively, a dry objective can be used.

2.1.6. Transfer the imaging box onto the microscope equipped with a dark climate chamber kept at 37 °C. Bring the objective to the CIW coverslip until the water drop touches it.

2.1.7. Using the epifluorescence mode, observe the tumor through the eyepiece and bring the cells into focus.

2.2. Time-lapse image acquisition

2.2.1. Select several positions of interest to image, and record their coordinates in the software. Ensure that the selected positions are representative positions from different sites of the tumor (each tumor can be different, but to ensure consistency, select the same amount of positions that are central to the tumor core and to the edges across all mice).

NOTE: A tile scan of a part of the tumor or of the whole visible tumor may be performed; however, if the tumor is large, this method will increase the time-lapse between images. In addition, if the tumor cells move fast, it can be challenging to track the same cells over time.

2.2.2. Switch to multiphoton mode and tune the laser to the correct wavelength. Note that, to avoid photodamage, higher wavelengths are desired.

NOTE: For Dendra2 imaging, a 960 nm wavelength was used. With the objective used in these experiments, a zoom of 1.3 was sufficient to get a good resolution of the tumor nuclei and scan a representative area of the tumor.

2.2.3. Go to the live mode and define a *z*-stack for each position in order to acquire the maximal volume of tumor cells without compromising the tumor cell resolution. Define the step size between the images as 3 µm.

2.2.4. Use the bidirectional mode to increase the scanning speed. The image resolution should be at least 512 x 512 pixels.

2.2.5. Acquire images of the tumor volume at different positions every 20 min for 2 h. Add water to the objective before each image acquisition.

NOTE: Time-lapse images can be automatically acquired by setting the right time-lapse in the software. However, the mouse can move, and the position or *z*-stack can shift over time, causing data loss. Therefore, it is recommended to perform the time-lapse manually and to check and adjust for *xyz* shifts in between acquisitions.

2.2.6. At this step, optionally, photo-switch the Dendra2 fluorescent marker.

NOTE: As opposed to time-lapse imaging (which allows studying individual tumor cells properties and how they change over time), this will allow studying the area of tumor cell infiltration in the

brain over several days[4]. A detailed explanation of this step is described by Gligorijevic et al.[13].

2.2.7. After the last image is acquired, remove the mouse from the stage and allow it to recover on a heating pad. To prevent dehydration, 100 µL of saline can be given subcutaneously. Place the mouse in its cage until the biopsy-like injury is performed.

3. Biopsy-like injury and CIW replacement

3.1. Create a biopsy-like injury at the tumor site 1 day after the first imaging session.

NOTE: Alternatively, another procedure, like partial tumor removal, can be performed.

3.1.1. Sedate the mouse using isoflurane inhalation anesthesia as previously described in step 2.1.1.

NOTE: While injectable anesthesia can be used, this procedure is shorter than the CIW implantation, and inhalation anesthesia makes it possible to precisely control and to reduce the time the mouse remains sedated.

3.1.2. Place the mouse on a stereotaxic frame and secure its head with two ear bars and a nose clamp. Check the depth of anesthesia by the lack of pedal reflexes. Use an anesthesia mask for stereotaxic surgery to keep the mouse sedated during the procedure.

3.1.3. Soak a cotton swab in acetone and spread it around the edge of the coverslip to soften the glue that holds the coverslip against the brain.

3.1.4. Slide thin point forceps under the coverslip to lift it. If the coverslip breaks at this point, use the forceps to remove the pieces of glass.

3.1.5. Apply cortex buffer to keep the brain moist.

3.1.6. Dip a 25 G needle in the tumor to a depth of 1 mm. Remove the needle and stop the bleeding, if necessary, by applying a gelatin sterile sponge.

NOTE: This step can be performed under a fluorescent stereomicroscope to more accurately identify the tumor area (if the tumor is too small). Additionally, a 1 µL solution of fluorescent polystyrene 1 µm beads can be injected during the puncture to identify the biopsied area.

3.1.7. Seal the brain surface with silicone oil and glue a 6 mm coverslip on the top.

4. Repeated imaging

4.1. One day after inflicting the biopsy-like injury (and on consecutive days if needed), repeat intravital imaging as described in Step 2 above to assess how the tumor cell behavior changes

over time. Select several positions of the tumor that would be representative of the whole tumor area.

NOTE: If fluorescent beads were used to identify the biopsied area, localize them to image tumor cell behavior in the biopsied regions compared to non-biopsied regions.

5. Image analysis

5.1. If the time-lapse images were acquired manually, combine them into one folder.

5.1.1. Open the time-lapse LIF file in the commercial software associated with the microscope (e.g., LasX or LAS AF). Select the tab **Process** > **Process Tools** > **Merge**. Select the first image of the time sequence and click **First**. Select the second image of the time sequence and click **Second**. In **Merge Dimensions**, select **t** for time. Click **Apply**. A new file with two timepoints will be generated. Repeat this process for all the timepoints, using the newly generated file as the first image of the sequence.

5.2. Correct the acquired *z*-stacks for any *xyz* shift.

NOTE: For the experiments described in this protocol, a custom-designed Visual Basic software program was used for correction. Alternatively, other available software can be used, such as ImageJ or Imaris.

5.2.1. Export the TIFF files from the software by right-clicking on the merged time-lapse file. Select **Save RAW Data**. The exported files will be exported to one folder named according to the channel, time, and *z*-position.

5.2.2. Open the TIFF files in the custom-designed Visual Basic software program.

5.2.3. Define the number of timepoints, *z*-stacks, and channels.

5.2.4. Assign a color to each channel by the order of appearance.

5.2.5. In the **Form Show** panel, for each timepoint, correct the shift in the *z* by clicking on the up (U) or down (D) buttons.

5.2.6. In the **Intravital image building** panel, select the channel to be used to correct for the *xy* shift. Select the green channel to correct, based on the signal from the tumor cells. Click **Automatic** for *xy* correction.

5.2.7. Export the corrected images as a maximum projection of three consecutive *z*-stacks. In the panel **Selection**, introduce the numbers of the first (**Begin**) and the last (**End**) *z*-slices to be exported. Select **Max**. Select **Separate folders for Z**. Click **Do**. For each of them, three *z*-stacks, time-lapse images will be exported to a separate folder.

5.3. Optionally, correct for the tissue elastic deformation.

NOTE: For tissue elastic deformation experiments, a match motion compensation software program was used to correct for rigid and elastic tissue deformation[14].

5.4. Track single cells in the complete *z*-stack in the time-lapse movie.

NOTE: Tumor cells can be tracked manually using, for instance, an ImageJ plugin (MTrackJ). While accurate, this is limited to the *xy* plane and is very time-consuming. Alternatively, tumor cells can be tracked automatically throughout the full *z*-volume, using tumor cell segmentation (e.g., Imaris software). However, in highly dense tumors, automated tracking is less accurate and can give rise to more tracking errors.

5.4.1. Open and track each time-lapse series (for three consecutive *z*-stacks) separately. Drag the folder containing the time-lapse images to ImageJ.

5.4.2. Select the tab **Plugins** > **Tracking** > **MtrackJ**. Track each individual cell by selecting **Add** and clicking on each cell at each timepoint.

5.4.3. Extract the measure of the tracks by clicking on **Measure** and saving the file.

REPRESENTATIVE RESULTS:

To assess the impact of biopsy on brain tumor cell behavior, we performed the procedure described in this protocol. Glioma—GL261 cells—expressing a nuclear fluorescent protein (H2B-Dendra2) were injected in the brain of C57BL/6 mice, and a chronic CIW was implanted. Time-lapse intravital imaging was performed on the same animal pre- and post-biopsy-like injury to the tumor (**Figure 1A,B**). The migration of individual tumor cells was determined by tracking the migration path over time in different *xy* planes of the *z*-stack (**Figure 1C**) and plotted as a percentage of migratory cells pre- and postbiopsy (**Figure 1F**). The tumor cell proliferation rate was quantified based on H2B-tagged Dendra2 condensation upon mitosis (**Figure 1D**) and plotted as a percentage of dividing cells pre- and postbiopsy (**Figure 1E**). We compared the distribution of migration velocity before and after biopsy in the same tumor and found that the number of migratory cells (velocity > 4 µm/h) increased after the intervention, with an associated decrease in the number of slow-/nonmigratory cells (velocity < 4 µm/h) (**Figure 2A**). On average per tumor, we observed a 1.75 (SD = 0.16)-fold increase in the percentage of migratory cells when a biopsy-like injury was performed, compared to control mice that were not biopsied (**Figure 2B**). We monitored tumor cell behavior for another week and found that, although the percentage of migratory tumor cells eventually decreased in both the control and the biopsied mice, the biopsied mice still exhibited a higher migratory capacity than the control mice (**Figure 2C**). The analysis of tumor cell proliferative behavior over time showed a 1.52 (SD = 0.26)-fold increase in the number of mitotic events upon biopsy, relative to nonbiopsied control mice (**Figure 2D**).

To test whether the observed effects of biopsy on tumor cell proliferative and migratory behavior

was an artifact due to CIW replacement surgery (required to perform a biopsy-like injury), we monitored tumor cell behavior in a group of mice that underwent CIW replacement without a biopsy. In this group, we did not observe any induction of migration or proliferation of tumor cells, indicating that the boost in tumor cell proliferation and migration rates were specifically triggered by biopsy-like injury (**Figure 3A, B**).

Stable photo-convertibility of the fluorescent protein Dendra2 allows for studying tumor cell infiltration over several days. Upon exposure to ultraviolet/blue light, Dendra2 is irreversibly switched from green to red. Using this property, a square region of the tumor was illuminated and ~200 Dendra2-expressing tumor cells were photo-marked before biopsy (**Figure 4**). One day after the biopsy, we relocalized the photo-switched region and measured the volume of tumor cells that had infiltrated into the surrounding tumor tissue. We found that the infiltration area was 1.72 (SD = 0.41) times larger in tumors after a biopsy-like injury compared to nonbiopsied control tumors (**Figure 4**). Although this approach only provides information on tumor cell bulk infiltrative behavior and not on a single cell level, it is less time-consuming than the time-lapse imaging approach and can be the method of choice for research questions focused exclusively on studying infiltrative behavior.

FIGURE AND TABLE LEGENDS:

Figure 1: Experimental setup for longitudinal intravital imaging of the biopsy effect on tumor cell behavior. (**A**) Diagram showing the design of the ring and the magnetic holder. (**B**) Schematic representation of the experimental workflow. Tumor cells are injected into the brains of mice and a CIW is established. Upon tumor development, a first (prebiopsy) time-lapse imaging session is performed. The next day, the biopsy and CIW replacement are implemented. The day after imaging (postbiopsy), a second time-lapse imaging session is performed. For long-term effects, subsequent imaging sessions can be done. (**C**) Images show representative snapshots of a time-lapse movie where GL261 H2B-Dendra2 tumor cells were tracked. Red lines depict individual tumor cell tracks. The scale bar = 50 µm. (**D**) Representative in vivo time-lapse images displaying dividing cells in GL261 H2B-Dendra2 tumors. Different stages of mitosis are indicated: prophase (P), prometaphase (Pm), metaphase (M), anaphase (A), and telophase (T). The scale bar = 50 µm. Graphs indicate the percentage of (**E**) migratory and (**F**) dividing cells pre- and postbiopsy. Each dot indicates the percentage of migratory cells in all the positions measured in an individual animal. The data are shown as mean ± S.E.M. of six mice (***P* < 0.01, paired *t*-test). This figure has been modified from Alieva et al.[4].

Figure 2: Representative results showing the impact of biopsy on tumor cell migration and proliferation rates. (**A**) Waterfall plots showing the change in cell velocity distribution relative to basal migration in individual mice. The data are shown as mean ± S.E.M. of five mice. (**B**) The number of migratory cells in control (blue) and biopsied (red) animals normalized to the number of migratory cells preintervention (*n* = 6 mice, ****P* < 0.0001, Student's *t*-test). (**C**) Tumor cell behavior was tracked over several days. Shown are the normalized (relative to preintervention) number of migratory cells in individual mice over time (*n* > 4 mice per condition, ****P* < 0.0001, two-way ANOVA). (**D**) The normalized number of dividing cells in control (blue) and biopsied (red)

animals. Per individual animal, the values postintervention were normalized to the values preintervention (n = 5 mice, $^{**}P < 0.01$, Student's t-test). This figure has been modified from Alieva et al.[4].

Figure 3: CIW replacement has no effect on tumor cell behavior. Longitudinal intravital imaging shows that the replacement of the CIW without biopsy has no effect on migration and proliferation rates. (**A**) The increase in the number of migratory cells for the indicated conditions. Every symbol represents the mean of an individual mouse, and $n \geq 4$ mice. (**B**) The increase in the number of proliferating cells for the indicated conditions. Every symbol represents the mean of an individual mouse ($n \geq 4$ mice, $^{**}P < 0.01$,$^{***}P < 0.001$, ns = nonsignificant, one-way ANOVA with Newman-Keuls post hoc test). This figure has been modified from Alieva et al.[4].

Figure 4: Diagram showing the experimental setup and representative results obtained with Dendra2 photo-switching. To monitor tumor cell infiltration upon biopsy, Dendra2-expressing tumor cells are photo-switched in a square region by UV/blue light illumination and imaged, 1 day before the biopsy. One day after the biopsy, the photo-switched region is relocalized and reimaged. Shown are representative Dendra2 images of tumor cell infiltration, corrected using channel subtraction. The white dotted line represents the infiltration area. The scale bar = 50 µm. The graph shows the increased photo-switched area plotted for biopsied (red) and control (blue) mice. Every dot represents the mean value of an individual mouse ($n \geq 5$ mice, $^{*}P < 0.05$, Student's t-test). This figure has been modified from Alieva et al.[4].

DISCUSSION:

Here we describe a method to study changes in tumor cell behavior at single cell level in response to invasive surgical procedures, such as a biopsy, in the brain of a living animal. The combination of longitudinal multiphoton imaging with the surgical implantation of a chronic CIW enables the quantification of tumor cell migration, invasion, and proliferation before and after biopsy in the same animal[4]. Compared to other approaches used for tumor multiday monitoring, such as bioluminescent imaging[12], MRI[10], or PET/CT[11], this method uniquely visualizes tumors on a single cell level and, thus, provides insight in cellular behavior underlying tumor progression.

To successfully perform this method, several procedures should be mastered. The most critical steps of this protocol are CIW implantation and replacement. The technical complexity of these steps requires precision and surgical skills that can be acquired with steady training. Complications during CIW surgery, such as bleeding which may cover the brain surface, may prove challenging for subsequent imaging. A lack of sterile tools or environment, as well as the failure to completely seal the brain surface, may cause an infection on the brain surface (white liquid under the coverslip), which will make imaging problematic and strongly compromise the resulting interpretation. Another common issue of this protocol is animal movement during the time-lapse imaging. While any *xyz* shift can be corrected after the experiment, it is recommended to correct the coordinate of each position before each time-point to prevent any loss of information. Tissue deformation is an additional problem found when imaging on an inverted microscope. Brain tissue suffers from compression when the mouse is placed in supine position. Depending on the degree of tissue deformation, tumor cell tracking may lead to an erroneous

quantification of cell displacement. To prevent this, a software for rigid and elastic deformation may be used[14].

While this procedure offers a broad application for studying changes in tumor behavior, certain limitations should be considered. This method allows scientists to image up to a depth of 1.6 mm (with the use of an optical parametric oscillator); however, this means that imaging is restricted to superficial brain cortex areas[15]. Thus, some brain tumors located in deep brain structures, including diffuse intrinsic pontine gliomas located in the brainstem region, cannot be studied in their original brain environment with this protocol. Another limitation of this protocol is the volume of the tumor that can be imaged. Although total tumor volume scanning is desired to obtain maximal information, often, tumor size and the speed of migratory cells can be limiting factors. For each tumor type, an optimal time-lapse for imaging has to be considered. If the time frame between images is too long, it may be difficult to track the tumor cells. The use of a resonant scanner can highly decrease scanning time, allowing the imaging of a bigger tumor[16]. Finally, the manual image analysis of this protocol can be very time-consuming, so instead, programs for automated 3D tracking can be used. However, the outcome of tracking should always be visually supervised since algorithms for automated cell tracking are rarely designed to recapitulate exactly the migration of the cells of interest.

Slight adaptations of the protocol described here can enable an even wider range of applications. Instead of performing biopsies, other (surgical) interventions may be implemented, such as partial tumor resection or the delivery of chemotherapy wafers. The addition of compounds through a surgically implanted microtube may be combined with this protocol to pharmacologically target specific molecules of interest. We expect that this model will be useful in studies aiming to analyze the impact of a certain intervention on tumor cell behavior. The possibility of performing repeated measures in the same animal not only provides more accurate data on changes occurring in the tumor but also greatly reduces the number of experimental animals needed per study.

ACKNOWLEDGMENTS:
The authors thank Anko de Graaff and the Hubrecht Imaging Center for their imaging support and Ellen Wehrens and Hannah Johnson for proofreading and editing the manuscript.

DISCLOSURES:
The authors have nothing to disclose.

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Figure 1

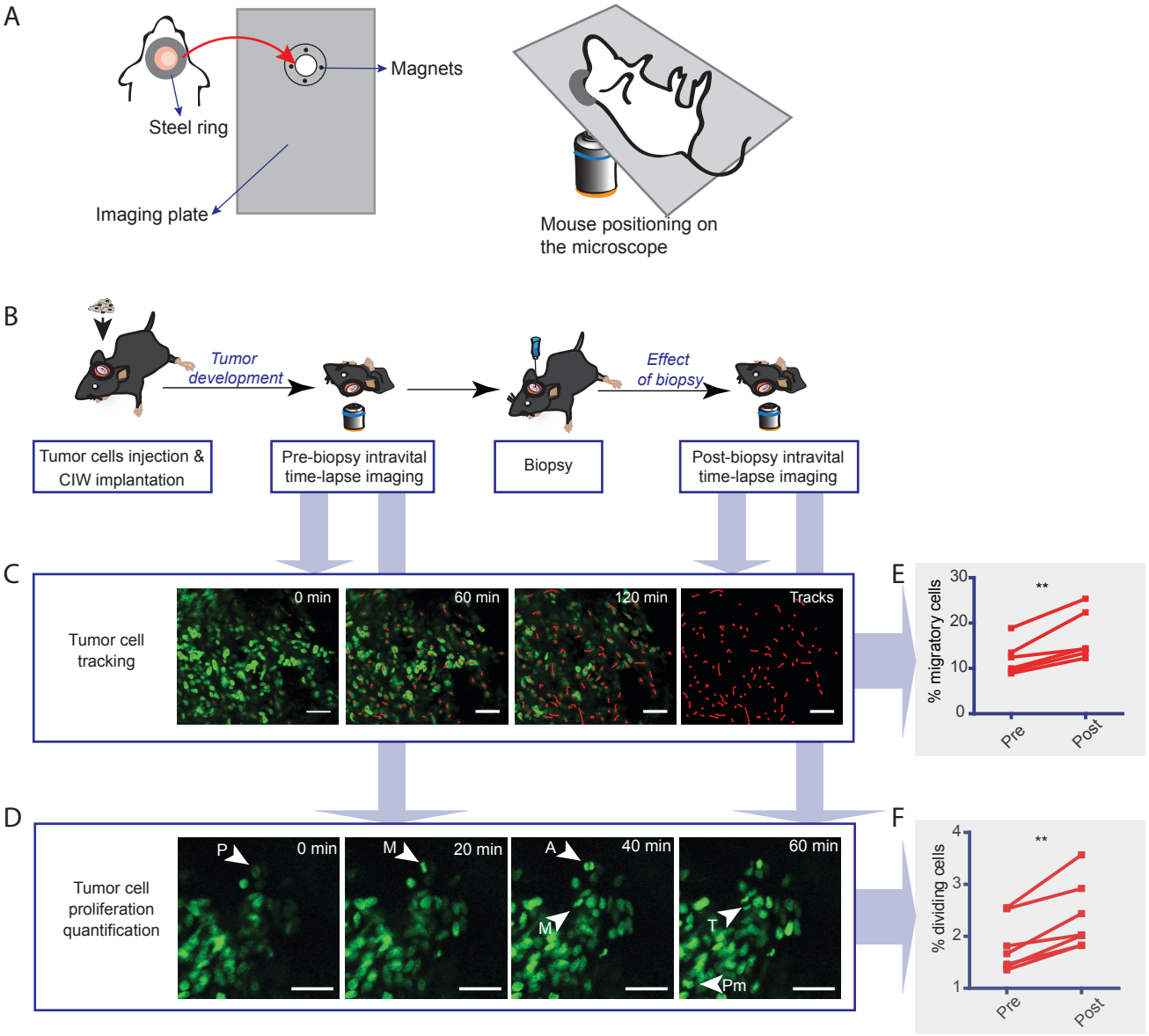


Figure 2

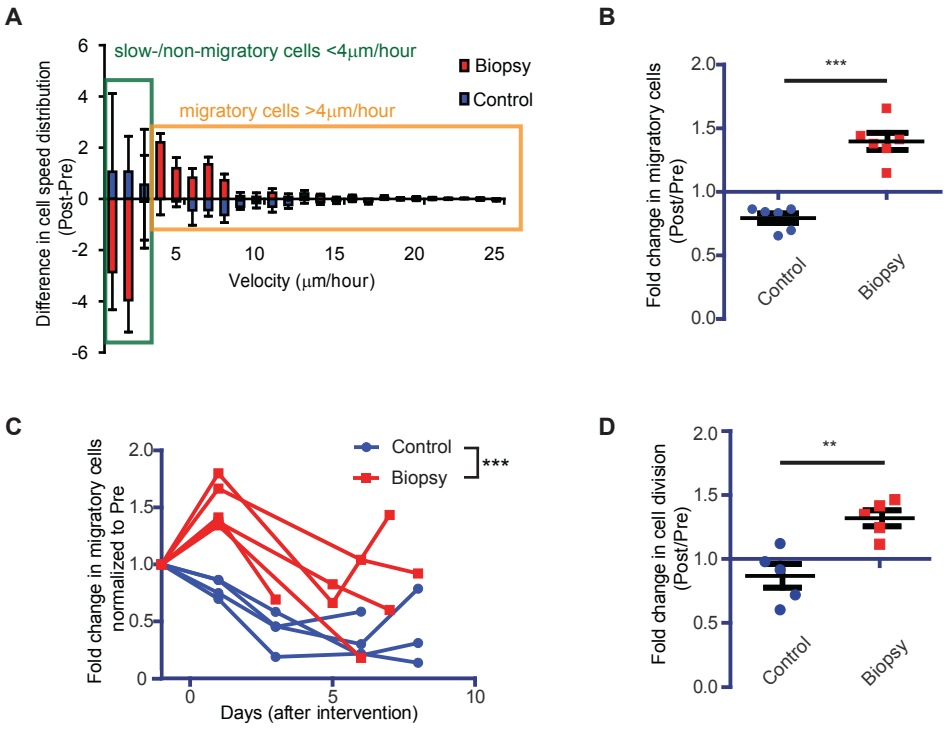


Figure 3

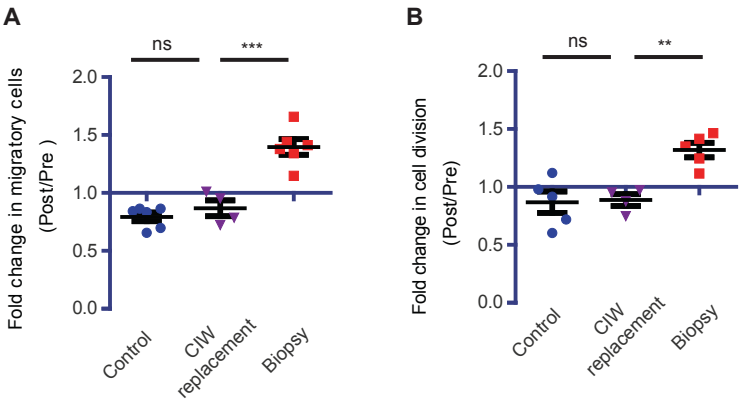
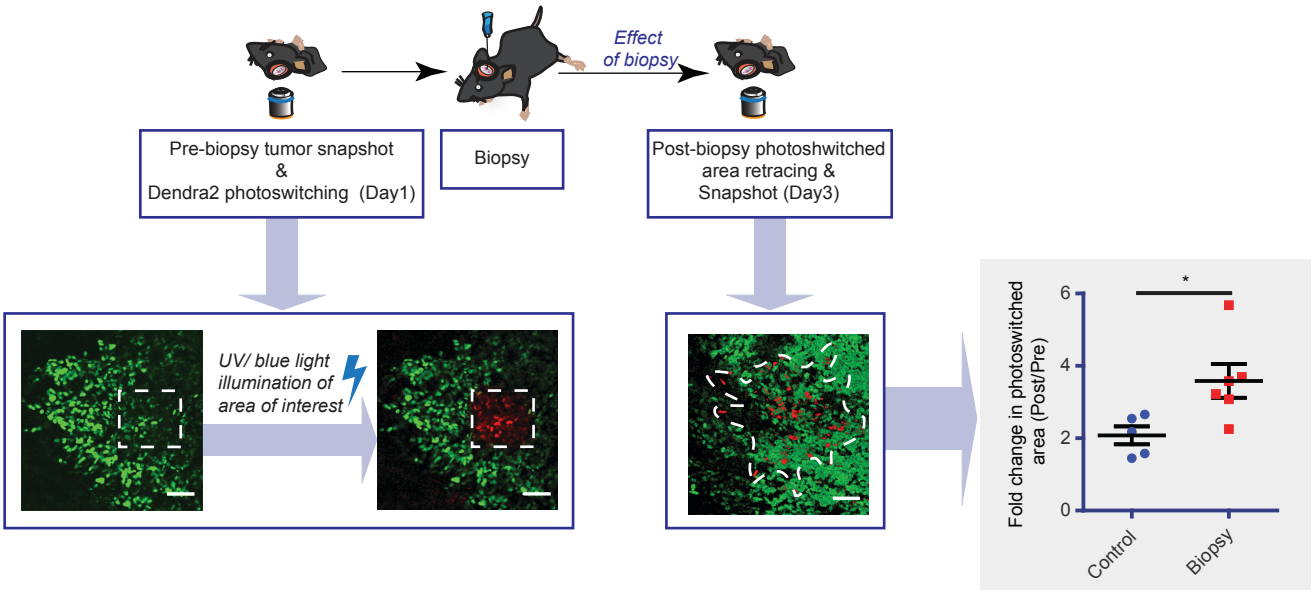


Figure 4



Name of Material/ Equipment	Company
25g x 16 mm hypodermic needles	BD Microlance
701 RN 10uL SYR W/O NEEDLE	Hamilton
Absorbable gelatin sponge	Pfizer
Coverslips round 6 mm	VWR international
Cyanoacrylate glue	Pattex
Dental cement	Vertex Dental
Drill	Dremel
Fine curved Tweezers	Dumont
Hypnorm	VetaPharma Ltd
Midazolam	Actavis
Opthalmic ointment	Kela Veterinaria
Quintessential Stereotaxic Injector (QS	Stoelting
Silicone Oil	Sigma Aldrich
Stereotaxic frame	Stoelting
Surgical stereo microscope	Olympus
Temgesic (0.3 mg/ml)	BD Pharmaceuticals
Vannas Tübingen Spring Scissors	Harvard Apparatus
Xylocaine (Lidocaine 1% + Epinephrine	Astrazeneca

Catalog Number

300600

7635-01

Gelfoam

631-0168

Pattex Ultra gel

Vertex Self-Curing

Dremel 3000 (dental drill may be more convenient) + 105 Engraving Cutter

AGT508

Hypnorm (Fentanyl citrate 0,315 mg/ml+ Fluanison 10 mg/ml)

Midazolam Actavis 5mg/ml

Duodrops veter kela 10 m

53311

181838

Lab standard stereotaxic, rat and mouse

283732

72-8508

Xylocaine (Lidocaine 1% + Epinephrine 1:100,000)

Comments/Description

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Point-by-point response to the editorial and reviewers comments

Editorial comments:

Changes to be made by the author(s) regarding the manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We are grateful for this advice and the revised version of the manuscript has been proofread by a native speaker.

2. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

A link to the editorial policy stating the permission to reprint has been added to the Editorial Manager. In the revised version we have included the citation in the figure legends.

3. Please upload each Figure individually to your Editorial Manager account as a .png, .tiff, .pdf, .svg, .eps, .psd, or .ai file.

In the revised version Figures are uploaded as ai. Files.

4. Please provide an email address for each author.

Email for all authors is provided in the JOVE submission website. Do we need to provide all emails in the manuscript as well? If so, where should it be placed?

5. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc.

Correct abbreviations are used in the revised version.

6. Please include a space between all numerical values and their corresponding units: 15 mL, 37 °C, 60 s; etc.

In the revised version we included a space between all numerical values and the units.

7. Please use the period symbol (.) for the decimal separator (i.e., 1.5 instead of 1,5).

In the revised version we used a period symbol for decimal separator.

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In the revised manuscript we have removed commercial terms and substituted them by generic terms.

9. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, dashes, or indentations.

In the revised version we have adjusted the numbering in the Protocol.

10. Please add more details 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. 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. See examples below.
In the revised version we provide additional details on the protocol steps as required in the comments below.

11. Lines 84-87: Please specify the age, gender and strain of mouse. Please mention how to confirm that the mouse is sedated. Please specify the concentration of isoflurane used for anesthesia.
In the revised version we included this information.

12. Line 92: Please specify the surgical marks and surgical equipment used.
In the revised version we indicate the area of the head that was shaved.

13. Line 97: What is used to glue skin edges to the skull?
In the revised version we specify the glue used to glue the skin edges to the skull.

14. Line 111: How about some guidance on what "desired amount" is?
In the revised manuscript, we specify the amount and type of tumor cells that was used in the experiments described in this protocol.

15. Lines 155 and 161: Please specify the concentration of isoflurane used for anesthesia.
In the revised manuscript we specify the amount of isoflurane used for the procedure.

16. Line 176: Please specify the positions that are selected in this step.
In the revised manuscript we explain how to choose positions of interest.

17. Lines 237-244: Software steps must be more explicitly explained ('click', 'select', etc.). Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc.).
In the revised manuscript we have included a detailed explanation of image processing and analysis.

18. Please include single-line spaces between all paragraphs, headings, steps, etc.
In the revised manuscript we have included single –line spaces.

19. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
In the revised manuscript we have highlighted the parts of the Protocol that should be included in the video.

20. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Please do not highlight any steps describing anesthetization and euthanasia.
In the revised manuscript we have highlighted the parts of the Protocol following these instructions.

21. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.
In the revised manuscript we have highlighted the parts of the Protocol following these instructions.

22. References: Please do not abbreviate journal titles.
For the first version the JOVE format of referencing was used, by default it abbreviates the journal titles. Could you please indicate which would be the correct format to use?

23. Table of Materials: Please remove trademark (™) and registered (®) symbols. Please sort the items in alphabetical order according to the name of material/equipment.
In the revised manuscript we have removed the above cited symbols and sorted items according to the name of the material.

Reviewers' comments:

Reviewer #1:

The manuscript by Alieva and Rios is a method for high resolution time-lapse multiphoton imaging of brain tumor cells before and after invasive surgical intervention (such as biopsy) within the same living animal.

This protocol expands upon another protocol already published in JoVE by Mostaney et al. (https://www.jove.com/video/680/a-craniotomy-surgery-procedure-for-chronic-brain-imaging). New in this manuscript is a protocol for injecting tumor cells in the brain during window implantation, a new preparation suitable for use on inverted microscopes, and a protocol for removing the coverslip after initial implantation so as to allow brain biopsies to be acquired.

In all, the protocol describes the window implantation technique accurately and well, but there are a number of deficiencies in the rest of the manuscript that need to be addressed before the protocol is ready for publication. The most serious of these is that the description for how data is acquired and analyzed, that is, how fields of view are chosen and the method of quantification is missing from the figure legends. This makes the plots difficult to understand, or in cases incorrectly labeled.

Finally, the manuscript would benefit from proofreading by a native English speaker as there are numerous grammatical errors throughout.

In summary, I can recommend this manuscript for publications after revision of the detailed issues listed below.

Answer:
We thank the reviewer for his/her positive comments and support of our manuscript. Below we addressed and clarified the constructive remarks of the reviewer.

Comment 1
-Line 88: Should monitor the mouse vitals with a pulse oxymeter such as Kent Scientific MouseStat, here and during imaging.

We thank the reviewer for this comment and have now added this as an optional step in our protocol. However, our extensive experience with this technique has shown that mouse vitals remain stable over the procedure and we have not suffered from loss of mice during the process.

Comment 2
-Line 97: Need to specify what glue to use (part number and manufacturer).
We thank the reviewer for this comment and have included the type of glue to use in the revised manuscript.

Comment 3
-Line 100: Need to describe how to tell how deep to drill. This is a critical step and needs to be described much more fully.
In the revised version we explain in more detail how to perform the drilling.

Comment 4
-Line 130: Using a #0 coverslip will introduce a large amount of spherical aberration if this objective lens is not designed for it. (See the point about the objective lens). Need to mention that the correction collar must be set correctly for this cover slip thickness, and how to do that. EMS offers 5mm and 8mm 1.5 coverslips which would be better suited for this objective lens. P/N 72296-05.
Please see below comment 8. The objective used for this experiments is collar corrected preventing spherical aberrations.

Comment 5
-Line 134: Please include a mechanical design drawing of the ring and the magnetic holder. This is crucial for people who have an inverted scope.
We thank the reviewer for this comment and have included a drawing of the ring and the holder in Figure 1 in the revised manuscript.

Comment 6
-Line 143: Inject how? Subcutaneously? Where?
We thank the reviewer for the comment and in the revised version we indicate that the buprenorphine was given subcutaneously.

Comment 7
-Line 155: It is critical to monitor the vital signs of a mouse under anesthesia, and required by many IACUC boards. Should include a couple of lines about heating the mouse and monitor the vitals here (see comment for line 88).
We thank the reviewer for the comment. Our experience shows that monitoring breathing visually was sufficient, since the dose of anesthesia used for intravital imaging is quite low. While in the first experiments we monitored body temperature with a probe, we found that the temperature was very stable over the experiment and did not use the probe in subsequent experiments. However, in the revised version we now include this as an optional step.
Comment 8
-Line 165: Please include a part number for the objective lens. The listed lens does not appear to be listed in Leica's catalog. The closest is a HCX IRAPO NA1.0 WD 2.6mm lens. This will also determine what is the correct cover slip to use.

The number of the provided objective is correct. Please find it in this brochure of Leica: https://www.leicamicrosystems.com/fileadmin/downloads/Leica%20TCS%20SP8/Brochures/Leica%20IR%20Objective-Flyer_EN.pdf

Comment 9
-Line 189: Need to add a discussion of the appropriate choice for zoom factor and laser power. Need to describe how laser induced tissue damage is evaluated and avoided.
In the revised version we have specified that higher wavelengths are desired to avoid photodamage. Also, the choice of the zoom factor should be made as a compromise between cell resolution and scanning area and we explain this in the revised manuscript.

Comment 10
-Line 216: It seems like this procedure may lead to acetone wicking under the coverslip and touching the brain tissue. Describe how it is possible this or why it is not a problem.
We found that by applying the acetone by means of a cotton swab we could prevent the acetone from leaking under the coverslip. The small amount of acetone is sufficient to soften the glue and the glass can be removed by the help of thin point forceps. To avoid misunderstanding we have rephrased this step of the procedure in the revised version of the manuscript.

Comment 11
-Line 218: It seems that breaking the coverglass could lead to shards of glass damaging the brain tissue. Need to describe how this is avoided.
Typically the coverglass breaks in two or three pieces that stay on top of the brain and do not damage it. In our manuscript we explain that the pieces are collected with forceps.

Comment 12
-Line 224: Include a part number and manufacturer for the beads in the materials list and here. Include details on the bead injection. How are the beads prepared, how are they injected and how much should be injected? This step should be recorded to show the procedure and the result as seen through the microscope shown. Given that the main novelty of this protocol over prior JoVE publications is imaging the response of damage, relocalization is a critical step.
In the experiments described in this protocol we did not use relocalization, but instead representative positions of the tumor were imaged before and after biopsy. We provide the use of beads as an optional step if there is a need for relocalizing the biopsy site. While the relocalization step may be of added value, it is not a critical step since our experiments show that by imaging different areas of the tumor change in tumor cell behavior can already accurately be detected. Therefore, we shortly describe bead-mediated relocalization of the biopsy site as an optional step in the revised version and indicate the amount of solution that is recommended to inject.

Comment 13
-Line 226: Missing a part number for the silicone oil from the materials list. Also Aldrich is spelled wrong.

We thank the reviewer for this remark and the revised manuscript we corrected the typo and provided the number of the silicone oil.
Comment 14
-Line 229: Need a description of how fields of view are chosen. Are the exact same fields chosen for imaging both before and after biopsy? If so, how are they identified? If not, then how are new fields

chosen after biopsy? How close to the biopsy site? Are fields both close and far from the biopsy compared? How far is close and far? These questions will determine if the effect is one of local damage, or of distant and systemic damage.
In the revised manuscript we have explained in more detail how positions are selected. In fact this is independent of the biopsy location (unless desired in the optional step where the biopsy site is located by fluorescent beads). Several positions that are representative for the whole tumor are selected. Note that the tumor at this stage is not large, therefore all positions are relatively close to biopsy sites.

Comment 15
-Line 231: This line is poorly worded, please check.
We have rephrased this sentence in the revised manuscript.

Comment 16
-Line 231: "trace" is the wrong word for this procedure. "Relocalize" is a better word.
We have used “relocalize” for this procedure.

Comment 17
-Line 231: As mentioned before, it is not clear if the same exact regions are being compared before and after biopsy.
In the revised version of the manuscript we have explained in more detail how regions were chosen in the second imaging session.

Comment 18
-Line 239: It would be useful to include their registration software as registration software for correction of z-shift is lacking from ImageJ and Imaris is very expensive.
In the revised version we explain in more detail how the correction was made using the custom-made correction software.

Comment 19
-Line 281: What were the conditions for photoconversion? Laser power at sample? Scan speed? Zoom factor? Number of scans?
As indicated in the manuscript this step is an optional step and explaining it in detail would make the manuscript too long. Moreover, we refer to another JOVE paper describing in detail how photoconversion is done (*Gligorijevic B et al*).

Comment 20
-Line 283: "Retraced" is not the correct word. Use "Re-localized" here.
We have used “relocalize” for this procedure.

Comment 23
-Line 308: The description of the plot is not clear. What is the vertical axis? Is this just the ratio of the numbers of cells?
It is indeed a ratio of the number of cells after intervention normalized to before intervention. In the revised manuscript we specify it in the legend.

Comment 24
-Line 332: "retraced" is the wrong word for this procedure. "Relocalize" is a better word.
We have used “relocalize” for this procedure.

Comment 25
-Line 333: It looks like channel subtraction was used here to remove the green signal from the photoconverted cells so that they appear more clearly converted and pure red. Image manipulations like this must be described in the -legend.
In the revised version we have explained that images were corrected using channel subtraction.

Comment 26
-Line 340: Why does the discussion come after the figure legends?
This follows the format provided by JOVE.

Comment 27
-Line 353: How can you tell if there has been infection? Need to describe this determination.
In the revised manuscript we specify how infection can be identified.

Comment 28
-Line 359: It is not accurate to call this a non-anatomical position. Perhaps say, "when the animal is in the supine position."
In the revised version we have rephrased this sentence.

Comment 29
-Line 359: It is not clear that the deformation that is observed is not due to bending of the coverslip. If there is a reference indicating that this is due to positioning of the animal, the authors should cite it here.
The visualization of the raw data shows that the deformation is due to tissue deformation. Since we take more z steps then just the tumor volume we can clearly see where the tumor tissue starts. This position does not vary overtime (something that you would expect with coverslip bending). However, overtime we can see that the tissue is expanding in the *xy* plane, indicating a deformation of the tissue due to tissue compression. A bended coverslip would lead to optical aberrations, something that we do not observe in our imaging data.

Comment 30
-Line 365: This is misleading as imaging to 1.6 mm requires specialized equipment like OPOs. The depth of imaging attainable by ordinary equipment should be listed and if desired, the 1.6mm depth mentioned in conjunction with the need for an OPO.
We have included this specification in the revised manuscript.

Comment 31
-Figure legends: The plots need to be better described. For example, in Figure 1D, the vertical axis seems to be mislabeled. It says % of migratory cells implying that the number of migratory cells is the denominator, but that is most likely not correct. This appears to be % of tumor cells that are migratory which is the number of migratory cells divided by the total number of tumor cells. Also, is this from a single field of view, or from multiple? These questions apply to just about every plot in the manuscript. Much more detail on how each plot was calculated needs to be included. This includes what the error bars signify in each plot (SD or SEM).
The plot represents the % of migratory cells in the tumor of individual mice, meaning how many tumor cells were migratory. Since we do not track other cells then tumor cells in this experiment, it is clear that we refer to tumor migratory cells. This is calculated based on all the tracked cells in all the

positions. As indicated in the legend the values are mean ± SEM. We clarified it in the revised manuscript.

Comment 32
-Figure 2B&D & Figure 3: It is not clearly described why the control group has a mean that is statistically different from 1. This seems to imply that the window implantation technique induced tumor cell motility and division that is in the process of recovery. This should be described in the manuscript and discussed as a source of error. Alternatively, the authors should have waited longer before imaging. There should be discussion of this in the manuscript.
Indeed, in the control group the % of migratory cells decreases overtime, however this is not due to the imaging window implantation (which was done at the same time as the cell implantation). This is just a consequence of how the analysis is done. Since the measure that we are presenting (fraction of the cells that are motile) is a relative value (to the total amount of cells) it is affected by the total amount of cells varying overtime. The tumor is growing over time meaning that after sham intervention there are more cells in the tumor. Therefore, the proportion of migratory cells over the total amount doesn't stay stable over time, which explains the percentage of migratory cells decreasing.

Comment 33
-Figure 2C: The presentation of so many lines in the plot is confusing and obscures the message of the data. The data for each category should be averaged and presented as a single line with error bars. Also, if there were four animals in each category, as the legend states, why are there five lines in the biopsy group?
In figure 2C we aimed to show tumors that were imaged over several consecutive sessions. However not all the mice were imaged during the same amount of time or on the same days, thus averaging is not an option. We find that even though there are many lines in the plot the result can easily be interpreted since the mice belonging to each group (biopsy and control) nicely cluster together.
Related to the reviewers question on the amount of mice and lines. The legend indicates: $n >= 4$. This means that each group had 4 mice or more, with 4 mice in the control group and 5 mice in the biopsy group.

Reviewer #2:
Manuscript Summary:
The manuscript (and protocol) is important for preclinical studies of the effect of tumor biopsy (and resection) on tumor cell migration in the (tumor) microenvironnement. Intravital microscopy is the only way, to have access to the microscopic level in vivo.

Answer:
We are grateful for the reviewer's time to evaluate our manuscript and for the acknowledgement of the relevance of our protocol for preclinical studies. Please see below how we have addressed the raised issues.

Major Concerns:
No major concerns

Minor Concerns:
Comment 1
-Line 92 : Put (e.g. Vannas Tübingen Spring Scissors) right behind: Use sharp scissors ().

We thank the reviewer for the comment and in the revised version we have moved this term.

Comment 2
-Line 97: Which glue?
In the revised version, we have indicated that cyanoacrylate glue was used.

Comment 3
-Line 122-123: Apply a drop of cortex buffer in the small space created in the brain before injection the cell suspension?
The cortex buffer is applied after the start of the cell injection. To clarify this point we moved this protocol step to a separate sentence in the revised manuscript.

Comment 4
-Line 175 and hereafter: information is missing on the laser? Please indicate the type of laser, excitation wavelength is 940 nm for dendra2 (is before photoswitch?), what is the laser power density at the entrance of the mouse cortex in mW/cm2?
We have now indicated the type of laser used in these experiments. The excitation is only indicated before photoswitching, since photoswitching is an optional step. The laser power would vary over days and sets of experiments, since it depends on the size of the tumor and how deep it was located.

Comment 5
-Line 280: what is the exact wavelength for photoswitch of dendra2?
For the photoswitched Dendra2 a 1020nm wavelengths can be used. All the steps referring to Dendra2 photoswitching experiments can be found in a previous paper that extensively explains the procedure (cited in the manuscript).

Comment 6
-Line 365: Two-photon excitation until a depth of 1.6 mm in a mouse brain is exaggerated. In optimal excitation condition the maximum excitation depth is in between 0.8 and 1 mm depending on the age of the animal: how younger how deeper.
While we agree with the reviewer that most optimal imaging is done at some superficial areas, it is possible to image up to a depth of 1.6mm, as indicated in the referenced paper. We feel it would be misleading to ignore this data.

Comment 7
-Fig 2B is not clear. First, the fold change in migratory cells for the control group is less than 1, this means a decrease of the migration velocity (µm/h)? In comparison to the biopsy group, you expect cell migration in the control group as well? Figure 2B is at what time after intervention?
The decrease in fold change means that there is a decrease in proportion of cells that are migratory over a time of two days (mice were imaged at day 1 and 3). This is just a consequence of how the analysis is performed. Since the measure that we are presenting (fraction of the cells that is motile) is a relative value (to the total amount of cells) it is affected by the total amount of cells varying over time. The tumor is growing over time, meaning that after sham intervention there are more cells in the tumor. Therefore, we detect that the percentage of migratory cells decreases as a proportion of the increasing number of total cells over time. It is important to note that in the experiments presented here we used a glioma cell line that is very migratory, so in the control group there are also migratory cells. What our results showed was that biopsy increased even further the proportion of migratory cells. In figure 2B we show the values one day after intervention.

Comment 8

-Why does biopsy increase migration? Biopsy disrupt the reactive gliosis around the tumor normally limits tumor cell migration? Please comment.

In our previously published paper (Alieva et al., Scientific Reports, 2017), we found that biopsy triggers monocyte recruitment to the site of biopsy, which in turn differentiate into macrophages. Macrophages are known to induce tumor cell migration and proliferation through chemokine and MMP secretion. Indeed, our results show that monocyte depletion could prevent biopsy-induced migration.

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