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TITLE:

Longitudinal Intravital Microscopy using a Mammary Imaging Window with Replaceable Lid

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SUMMARY:

This protocol describes a novel mammary imaging window with a replaceable lid (R.MIW). Intravital microscopy after implantation of the R.MIW allows for longitudinal and multi-day imaging of the healthy and diseased mammary gland with a cellular resolution during the different developmental stages.

ABSTRACT:

The branched structure of the mammary gland is highly dynamic and undergoes several phases of growth and remodeling after birth. Intravital microscopy in combination with skin flap surgery or implantation of imaging windows has been used to study the dynamics of the healthy mammary gland at different developmental stages. Most mammary imaging technologies are limited to a time frame of hours to days, whereas the majority of mammary gland remodeling processes occur in time frames of days to weeks. To study mammary gland remodeling, methods that allow optical access to the tissue of interest for extended time frames are required. Here, an improved version of the titanium mammary imaging window with a replaceable lid (R.MIW) is described that allows high-resolution imaging of the mammary gland with a cellular resolution for up to several weeks. Importantly, the R.MIW provides tissue access over the entire duration of the intravital imaging experiment and could therefore be used for local tissue manipulation, labeling, drug administration, or image-guided microdissection. Taken together, the R.MIW enables high-resolution characterization of the cellular dynamics during mammary gland development, homeostasis, and disease.

INTRODUCTION:

The mammary epithelium is a unique secretory organ present in mammals, which produces and secretes milk to nourish offspring. Throughout life, the mammary gland undergoes multiple rounds of development and growth, which are accompanied by structural and functional changes of the tissue[1]. Depending on the developmental stage, the cell types contributing to tissue remodeling are different, as well as the location within the ductal tree.

Multiphoton intravital microscopy (IVM) allows the study of mammary cell dynamics *in vivo* in the native and minimally perturbed setting[2–4]. To obtain visual access to the mammary gland, several temporary *ex vivo* or skin flap imaging techniques have been published during different stages of mammary gland development, including puberty[4–7], adulthood[2,8], lactation[9–12], and tumor dynamics[13–15]. Although these techniques result in high spatially and temporally resolved imaging of mammary cell dynamics, the time frame is limited to hours. Because most of the mammary gland remodeling processes will take days to weeks, methods that allow optical access to the tissue of interest for extended time frames are required. Over the years, several permanent imaging windows have been developed for mammary tumor imaging[15–18], including a titanium mammary imaging window (MIW)[2,3,19]. Although very useful to study mammary tumor growth, visualization of the healthy mammary gland structure remained limited to a few days. Recently, a flexible silicon imaging window was developed, which allows visualization of the pubertal mammary gland for multiple weeks[20]. However, the mammary gland is embedded in an adipocyte-rich fat pad, which leads to extensive light-scattering and as a result limited visibility of the mammary ductal structures. Therefore, superior imaging conditions are required at all times to visualize tissue dynamics over prolonged periods of time in the mammary gland. Neither the classical MIW nor the flexible silicon window, allow for tissue manipulation or optimization of physical tissue location before imaging, as the window forms a closed system after surgery and implantation. As a result, optimal optical access to the underlying mammary tissue is likely to become precluded over longer time periods. In contrast, the skin flap technique does allow for optimization and repositioning of the tissue during the imaging session and a skin flap can be repeated multiple times[2]. However, repeated imaging sessions through a skin flap are only possible when sufficient time (at least 7 days) is allocated in between surgeries to allow recovery of the skin and is therefore mostly suited to study processes at longer time scales. Moreover, it is advisable to not perform this procedure many times because of its invasive nature and large risk of infections and scarring upon wound closure.

To overcome these limitations, i.e., to ensure optimal imaging conditions for a prolonged period of time at a high frequency, and at the same time allow tissue manipulation, an improved titanium version of the MIW with a replaceable lid (R.MIW) was designed to visualize the healthy and diseased mammary gland over multiple days to weeks[2] (**Figure 1A,B**). The R.MIW was custom designed to provide optimal tissue access, enabling direct tissue manipulation over the entire duration of the IVM experiment, and thereby permits visualization of the mammary gland at the right time and place over prolonged periods of time. When closed, the R.MIW forms an airtight system comparable to the classical MIW (**Figure 1C**). When opened under aseptic conditions, the R.MIW allows local tissue manipulation to improve optical access and also enables local administration of substances, such as pathway inhibitors or agonists, injection of different cell

types of interest, such as cancer cell or immune cell populations, or tissue labeling-dyes. The lid can be opened at any moment in between imaging sessions without causing any damage to the underlying tissue.

[Place **Figure 1** here]

This protocol describes the design and implantation procedure of the R.MIW, as well as a longitudinal IVM strategy to revisit the same mammary ducts and their visualization at a cellular resolution. The R.MIW allows following cell divisions and morphological changes during different developmental phases of the mammary gland in diverse fluorescent reporter mouse models. Taken together, the R.MIW facilitates high-resolution characterization of the cellular dynamics during mammary gland development, homeostasis, and disease.

PROTOCOL:

All procedures described in this paper were performed in accordance with the guidelines of the IACUC and KU Leuven and were carried out within the context of approved project application P160/2020. Review the analgesia strategy with the institutional veterinarian and administer pre- and post-operative analgesia as per institutional guidelines.

1. Preparation of the R.MIW (estimated timing: 2 h)

NOTE: For the construction of the R.MIW, it is advisable to find a local manufacturer who specializes in working with hard materials such as titanium, as this requires special tools and expertise. Workshops specializing in the design and manufacture of titanium prosthetics usually have the machinery and expertise to produce the R.MIW parts.

1.1 Place the lid of the R.MIW on a sterile surface with the outside of the lid facing upwards (and the arms of the lid sloping downwards; **Figure 1A**).

1.2 Apply cyanoacrylate adhesive glue around the entire rim lid perimeter and carefully place a 10 mm round glass coverslip on top of the lid.

NOTE: Always prepare a spare lid with a glass coverslip as a backup during the surgical procedure.

1.3 Use a sterile wooden stick to position the glass coverslip and apply some force to push it down to ensure an air-tight seal between the glass coverslip and the frame of the lid. Make sure the holes in the arms of the lid are not covered up by the glass.

1.4 Disinfect the lids and the R.MIW by submerging in 80% ethanol for at least 30 min in a closed Petri dish.

NOTE: Do not leave the lids longer than 1 h in 80% ethanol to avoid the dissolution of the cyanoacrylate adhesive glue.

1.5 Remove the excess cyanoacrylate adhesive glue on the glass coverslip by using a cotton tip soaked in acetone.

1.6 Store the lids and the R.MIW in a sterile environment until further use.

NOTE: The R.MIW frame and lids can be kept in a sterile tube or bag for a maximum of 1 week. When resuming the experiment, always examine whether the glass coverslip is still properly attached to the lid prior to proceeding with the protocol.

2. Preparation for surgery (estimated timing: 15 min)

NOTE: For the R.MIW implantation procedure, female mice (>3.5 weeks old) of any strain were used.

2.1 Maintain sterility by using sterile gloves, and sterilize all items that will come in contact with the mouse. Wash surgical tools with water and mild soap, air dry, wrap in aluminum foil with no exposed corners or edges, and sterilize using an autoclave for a cycle of 20 min at 120 °C prior to the surgery.

2.2 Prepare a sterile surgical platform and sterilize the surfaces with 80% ethanol.

NOTE: To ensure sterility, the surgery can be performed in a flow cabinet.

2.3 Turn on the heating pad and cover the mat with a sterile drape. Layout the sterile instruments on the sterile drape and place the R.MIW and a minimum of two glass-covered lids in sterile phosphate-buffered saline (PBS) in a Petri dish (close the lid to avoid external contamination).

2.4 Anesthetize the mouse in an induction chamber using a 3% isoflurane/O_2 mixture. Ensure that the animal is relaxed enough to be easily manipulated and transferred to the nose mask without any struggle.

2.5 Transfer the mouse from the induction chamber to an anesthesia nose mask and reduce the isoflurane level to 1.5% – 2% isoflurane/O_2 mixture. Verify full anesthesia by performing a paw withdrawal test.

2.6 To perform the paw withdrawal test, firmly pinch the animal paw using fingernails or blunt end forceps. In the absence of any muscle reflex, consider the animal as unconscious and proceed for surgical preparation. Perform this test before animal manipulation and repeat regularly during the anesthetic and surgical procedures to confirm anesthesia.

2.7 Apply eye ointment to prevent corneal dehydration.

2.8 Shave the area around the 4th mammary gland using a razor blade (**Figure 2A**). Use the 4th nipple as a landmark. Remove loose hairs using sticky tape.

NOTE: Alternatively, depilatory cream can be used to remove the hairs.

2.9 Disinfect the exposed skin with 80% ethanol or povidone-iodine, and position the mouse in the sterile surgical area on its back with the snout into an anesthesia nose mask using 1.5% isoflurane/O_2 mixture.

2.10 Secure the front and hind limbs of the mouse using paper tape.

NOTE: Leave the anesthetized and recovering mouse on the heating pad as much as possible, before and after surgery.

3. R.MIW implantation (estimated timing: 30 min)

3.1 Verify if the animal is adequately anesthetized by performing a paw withdrawal test. Gently lift the skin using a fine Graefe forceps and make a 10–15 mm diagonal incision using small spring scissors in the skin on top of the 4th mammary fat pad using the 4th nipple as an orientation point (**Figure 2A**). Make sure not to cut or damage the peritoneum or the mammary gland.

3.2 Define the region of interest (ROI) based on macroscopic features of the mammary tissue, including the location of the lymph node or a tumor lesion, and try to keep the ROI central with regard to the incision.

3.3 Separate the skin from the mammary fat pad and underlying tissue layers using blunt dissection of up to 5–8 mm all around the incision line to create a pocket to fit the R.MIW frame (**Figure 2BI**).

3.4 Pick up the R.MIW using sterile forceps and test whether the incision is large enough to insert the window. If needed, slightly enlarge the incision until the R.MIW fits in.

3.5 Remove the R.MIW and place a purse-string suture all around the incision using a 5-0 silk suture (braided, non-resorbable). Place the suture 1–2 mm from the edge of the incision from the outside to the inside of the skin, starting from the caudal end of the incision.

3.6 Move approximately 5 mm up along the incision and pass the suture thread through the skin from inside to outside. Repeat this procedure along the edge of the incision thereby creating a circular suture consisting of 5–6 loops (**Figure 2BII**). The final exit of the suture thread should be located approximately 2–5 mm away from the first entrance.

NOTE: Be careful not to place the suture too close to the edge of the incision, as this increases the risk of tearing the skin. Placing the suture too far from the edge of the incision increases the risk of infection between the excess skin and the groove of the R.MIW.

3.7 Fit the R.MIW inside the incision and use the Graefe forceps to carefully place the skin in the groove of the R.MIW. Make sure to leave the loops of the suture outside.

3.8 Pull on the loops of the purse-string suture and tighten the suture in the groove of the R.MIW by gently pulling on both ends of the suture (**Figure 2BIII**). Tie off with surgical knots and cut away the excess thread.

3.9 Apply petroleum jelly to the inner rim of the R.MIW using a toothpick, while making sure to avoid the underlying tissue. Gently push the window frame downwards with sterile forceps or two fingertips, and position the lid into the R.MIW frame (**Figure 1B**). This will avoid a volume of air in between the mammary gland tissue and the R.MIW lid.

3.10 Place the tips of thin forceps through the holes in the arms of the lid and tighten the lid by twisting the lid clockwise (approximately 5°) (**Figure 1C and Figure 2BIV**).

3.11 If some air remains after tightening the R.MIW lid, use a sterile insulin needle to remove the excess air. Introduce the needle through the skin into the cavity between the mammary tissue and the lid and slowly pull out the plunger, thereby creating a vacuum between the mammary gland tissue and the R.MIW lid.

3.12 Put the mouse back into the cage to recover and monitor closely until it is fully awake, or proceed with step 4 for immediate imaging.

3.13 Administer, post-surgery, analgesia such as buprenorphine (0.1 mg/kg diluted in sterile 0.9% NaCl) by subcutaneous injection once the mouse is removed from the imaging box and still under anesthesia. Repeat subcutaneous injection with buprenorphine at 8 h and 16 h after the surgery.

3.14 At this post-surgical stage, monitor the mice closely for signs of discomfort or complications based on vital parameters, such as respiration, reactivity, behavior, posture, and body weight. Monitor the skin surrounding the window carefully for signs of inflammation and necrosis. If no complications occur, the R.MIW will allow for repeated IVM sessions over several weeks post-implantation.

[Place **Figure 2** here]

4. Repeated intravital microscopy through the R.MIW

NOTE: The repeated IVM strategy described here was optimized for an inverted multiphoton confocal system equipped with a motorized stage and a dark climate chamber at 35.8 °C.

4.1 Anesthetize the mouse in an induction chamber using a 3% isoflurane/O_2 mixture. Verify loss of consciousness by performing a paw withdrawal test (described in step 2.6). Apply eye

ointment to prevent corneal dehydration during the imaging session.

4.2 Inspect the condition of the R.MIW frame and lid, including the stability of the sutures or any potential damage to the lid. In case of damage to the lid, the lid can be replaced as described in step 5.

4.3 Transfer the mouse to a pre-heated (37 °C), custom-made imaging box (**Figure 3A**) and place the mouse with the head into the nose cone. The imaging box consists of a frame that fits within the automated stage of the microscope. The frame is designed to hold a custom-made inlay with a hole the diameter of the R.MIW (**Figure 3A**)**.**

4.4 Connect the inlet with a nose cone to the front of the box and attach it to an isoflurane vaporizer station using 2%–3% isoflurane/O_2 mixture. Connect the outlet to an anesthetic scavenging unit to ensure circulation and to prevent the accumulation of isoflurane in the box. Close the imaging box using a transparent lid.

4.5 Position the mouse on the inlay in such a way that the R.MIW falls in the hole of the imaging box inlay (**Figure 3A**), and fit the imaging box in the stage of the microscope. Apply semi-transparent and paper tape over the back of the mouse to stabilize the mouse and reduce tissue motion due to respiration.

4.6 Reduce the isoflurane dose gradually over the course of the imaging session to sustain a stable, but superficial anesthesia (typically between 1.5%–0.8% isoflurane/O_2 mixture).

4.7 Provide proper nutrition during imaging as described below.

4.7.1 For imaging sessions <3 h, inject the mouse subcutaneously with 200–300 µL of sterile PBS for hydration.

4.7.2 For imaging sessions >3 h, follow the steps outlined below.

4.7.2.1 For long-term imaging, infuse the mouse with nutrients. For this, use a commercially available sterile infuse mixture containing amino acids and glucose. Place a flexible needle subcutaneously in the neck of the mouse and secure it with paper tape.

4.7.2.2 Attach a 10 mL syringe with the prepared nutrient mix to a flexible silicon tube and push the nutrient mix through until it has reached the end of the tube.

4.7.2.3 Connect the silicone tube to the flexible needle and inject 50 µL of infuse solution every 30 min.

4.7.2.4 Take the end of the tubing and place it through one of the holes of the imaging box (from inside to outside, leaving the needle attachment side in the box).

4.8 Secure the imaging box in the stage of the microscope and define the area of interest using the epifluorescence mode of the microscope.

4.9 Apply the desired microscope settings depending on the mouse model (**Figure 3B**). Vessel structures, collagen patterns (visualized by second harmonic generation), and other stable anatomical structures, including the inguinal lymph node, may be used as landmarks.

4.10 Acquire images using an inverted multiphoton confocal microscope at a 12-bit depth, and a 25x water objective using a Z-step size ranging from 1.0 to 4.0 µm (total z-stack ranging from 150 to 800 µm). Apply the following standard settings: 1024 x 1024 format, 600 Hz scan speed, bidirectional mode.

4.11 Visualize collagen I fibers by performing second harmonic generation by using an excitation wavelength of 860 nm and detection at 425–435 nm. Excitation and detection wavelengths for the different fluorophores are indicated in **Table 1.**

[Place **Table 1** here]

4.11 Use the **Spiral** function in imaging software navigator mode to generate a large overview tilescan to create a reference map of the tissue (**Figure 3B**). Define the ROIs within the spiral overview, define the upper and lower Z planes to determine the Z stack. Press **Start** to obtain detailed three-dimensional (xyz) or four-dimensional Z-stacks (xyzt) of the tissue.

NOTE: During the entire imaging experiment the mouse should be closely monitored. For long imaging sessions, a pulse oximeter and temperature probe are recommended. The breathing of the mouse should be regular and at the same pace. If the mouse starts to show signs of gasping, the amount of isoflurane should be reduced.

4.12 At the end of each imaging session, keep the mouse on a heating pad until fully awake. If the mouse shows signs of discomfort or reduced mobility, keep the cage for a longer time on the heating pad and provide nutrient gels instead of the regular chow.

NOTE: In between imaging sessions, the mouse should be closely monitored for signs of discomfort. Typical signs of discomfort in between IVM imaging sessions are decreased food and water consumption, rapid breathing, decreased movement, tremor, abnormal body posture, or unkept fur. In case of clear signs of discomfort, follow institutional guidelines regarding animal welfare and terminate the experiment when the humane endpoint is reached.

4.13 Repeat steps 4.1–4.11 for every repeated imaging session, and use the overview tilescan to retrace the same position(s) over multiple time points (**Figure 3B**). Imaging frequency will depend on the research question and the timing of the studied process, and typically varies from imaging sessions twice a day to once per week.

4.14 At the end of the desired experimental period, euthanize the animals with an implanted

R.MIW using deep anesthesia with isoflurane followed by cervical dislocation. If desired, dissect the organs of interest for further *ex vivo* analyses.

4.15 Alternatively, keep the animal alive for future *in vivo* analyses. In that case, remove the pouch suture holding the R.MIW by cutting it with spring scissors and carefully detach the window ring from the mouse skin using blunt forceps. Clean up the edges of the skin that previously held the window and proceed with a simple continuous suture (absorbable material, such as polyglycolic acid).

4.16 Once the skin is closed, tie the beginning and ending suture threads with two square knots (4 throws). To minimize tissue ischemia, the knots should be loose enough to allow blood flow at the skin edge. Disinfect the wound with povidone-iodine to prevent inflammation and to promote healing of the skin.

[Place **Figure 3** here]

5. Opening and closing the lid of the R.MIW in between imaging sessions

5.1 Maintain sterility by using sterile gloves, and sterilize all items that will come in contact with the mouse. Autoclave surgical tools prior to the surgery (see step 2 for details).

5.2 Turn on the heating pad and cover the mat with a sterile drape and anesthetize the mouse in an induction chamber using a 3% of isoflurane/O_2 mixture. Verify adequate anesthesia by performing a paw withdrawal reflex test.

5.3 Transfer the mouse from the induction chamber to an anesthesia nose mask and reduce the isoflurane level to 1.5%–2% isoflurane/O_2 mixture.

5.4 Place the tips of thin forceps through the holes in the arms of the lid and loosen the lid by twisting the lid anti-clockwise. If needed, apply some pre-heated sterile PBS (37 °C) on the window to facilitate the loosening of the lid.

5.5 Place the lid of the R.MIW in a sterile dish with sterile PBS until further use. Clean the window using demi-water and subsequently 80% ethanol. Keep the lid in sterile PBS until further use.

5.6 Prevent the exposed tissue from drying out by adding some drops of sterile PBS to the tissue surface.

5.7 Apply or inject any substance or cells of interest on the exposed tissue, a maximum volume of 50 µL can be used. Wait a few minutes until the liquid is absorbed by the tissue. Alternatively, the tissue can be manipulated or repositioned using sterile, blunt forceps to optimize the imaging conditions for specific ROIs.

5.7 Apply petroleum jelly to the inner rim of the R.MIW, making sure to avoid the underlying tissue. Position the lid into the R.MIW frame as described in step 3.9 and place the tips of thin forceps through the holes in the arms of the lid and tighten the lid by twisting the lid clockwise (approximately 5°).

REPRESENTATIVE RESULTS:

To study the proliferative dynamics of mammary epithelial cells during the different developmental stages of the mammary gland the procedure as described in this protocol was performed. The design of the R.MIW is depicted in **Figure 1**, and the procedure to implant an R.MIW is summarized in **Figure 2**. The R.MIW is surgically implanted between the mammary gland and the skin. A suture is used to hold within the window ring and prevent the mice from pulling on the sutures (**Figure 2**). The sutures holding the R.MIW are made of non-absorbable silk, which has hypoallergenic properties, a soft texture, and is easy to handle. The R.MIW ring is made of titanium, one of the most biocompatible materials that do not promote inflammation or necrosis after implantation[21]. If the aseptic conditions are followed and the sutures are well executed, the R.MIW mammary gland implantation poses a low risk of postoperative complications. Moreover, unlike abdominal imaging window implantation[22], R.MIW implantation is not a limiting factor for mouse survival because the mammary gland is not a vital organ and allows daily imaging up to the limit specified in the ethical protocol. The only factor that limits the duration of window implantation is the homeostatic turnover of the skin, which will eventually lead to the sutures falling out after 4 – 6 weeks. Therefore, it is important to regularly inspect the stability of the sutures. If desired, the sutures can be removed and replaced by a new purse-string suture under aseptic conditions (as described in steps 3.5 – 3.8) to reassure window stability.

A major challenge when using the multiday imaging approach is retracing an ROI on consecutive days. To this end, a quick overview scan can be included before selecting the ROI(s) (**Figure 3B**). Multiple tissue landmarks can be used to retrace the same region of the tissue, including the collagen network signal (visualized by second harmonics generation), tissue structure, as well as local patterns of cells differentially or stochastically labeled with dyes or fluorescent proteins (**Figure 3B**). In this representative example, an R.MIW was implanted onto a mammary gland in an *MMTV-PyMT;R26-CreERT2^{het};R26-Confetti^{het}* female mouse at the onset of palpable tumor formation. Subsequently, stochastic recombination was induced by injection of 1.5 mg tamoxifen, resulting in recombination of the Confetti construct in some cells (**Figure 3C**). The recombined tumor cells were followed for a period of 20 days (**Figure 3B,C**). If imaging of the same region of the mammary gland over consecutive days is precluded, the window can be opened in an aseptic environment (as described in step 5.1) to further clear up, and reposition the tissue to improve visibility and image acquisition (**Figure 3A**).

Using this method, the developing mammary gland during puberty was followed at the single-cell level. Repeated IVM through an R.MIW was performed using *R26-CreERT2^{het};R26-mTmG^{het}* female mice between 4–6 weeks of age, in which all cells are labeled with membrane tdTomato (**Figure 4A**)[23]. Mice were injected with a low dose of tamoxifen (0.2 mg/25 g body weight), resulting in sporadic recombination of the mTmG construct, causing a change from red to green

in some cells in all tissues, including the mammary gland (**Figure 4B**). The same ROIs were revisited over multiple days to visualize morphological changes within the mammary gland, including ductal elongation and ductal branching (**Figure 4C**) at a cellular resolution. Importantly, this combination of stochastic cell labeling and multi-day IVM also allows visualizing the dynamic changes of the ductal environment, such as the dynamics of single cells in the stroma surrounding the elongating and branching ducts (**Figure 4D**) as well as the cellular dynamics within the inguinal lymph node (**Figure 4E**).

[Place **Figure 4** here]

Similarly, in the adult mammary gland, the proposed R.MIW approach allows visualization of the same ductal structures over multiple days with uncompromised visibility. Even the use of less bright fluorescent reporter models, such as the *Cdh1-mCFP* (E-cadherin-mCFP) mouse model[24], permits visualization of ductal stability and subtle morphological changes at a cellular resolution (**Figure 5**). Note that visibility between day 3 and day 5 improved significantly after tissue repositioning (**Figure 5**).

[Place **Figure 5** here]

To assess proliferative heterogeneity of the mammary epithelial cells during the hormonal cycle at the single-cell level, the photo-convertible Kikume Green-Red (KikGR) reporter mouse model[25,26] was used, which has ubiquitous expression of the KikGR protein. KikGR is a bright fluorophore that undergoes green-to-red conversion upon exposure to violet light and the red/green ratio can be used as a proxy for the proliferative activity of a cell[2] (**Figure 6A,B**). Using the R.MIW approach, we followed the same cells within the ductal tree of the adult mammary gland over several days and found previously unanticipated proliferative heterogeneity throughout the ductal tree (**Figure 6C**). High and low proliferative cells were equally distributed over the different converted areas (**Figure 6C,D**). Strikingly, at the local level, neighboring cells showed large differences in their red/green ratio (**Figure 6C,D**). Quantification of the red/green ratio of different cells (as a proxy for their proliferative activity) 10 days after photo-conversion, revealed highly variable dilution rates of some cells within the same ductal micro-environment (**Figure 6E**). Together, these data reveal a remarkable local proliferative heterogeneity within the adult mammary gland, and at the same time, a global uniform turnover rate.

[Place **Figure 6** here]

FIGURE AND TABLE LEGENDS:

Figure 1: Design of the mammary imaging window with a replaceable lid. (**A**) Top view and side view of the replaceable lid of the mammary imaging window, with a 10 mm coverglass glued to the ring. (**B**) Top view and side view of the mammary imaging window, which consists of an outer ring and inner ring, with a groove in between to secure the window within the skin of the mouse using a purse-string suture. The outer ring has a small groove that fits the four projections (arms) of the lid. (**C**) Cartoon and pictures demonstrating the opening and closing mechanisms of the lid. The slanting projections make sure the lid is fixed inside the window frame. This figure has been

modified from Messal et al. [2].

Figure 2: Overview of the surgical procedure and window implantation. (**A**) A diagonal incision is made near the 4th nipple of a female mouse and the skin and underlying tissue are disconnected by blunt dissection. (**B**) The inguinal lymph node and shape of the fat pad can be used for the right orientation of the tissue (I). A purse-string suture is placed in the skin around the incision (II), and after fitting in the window frame, the suture is tightened in the groove to secure the window frame, and secured by surgical knots (III). The last step is to insert and lock the lid into the window frame (IV).

Figure 3: Longitudinal IVM workflow. (**A**) Schematic depiction of a typical multi-day IVM experiment. (**B**) Imaging of a tumorigenic region within the adult mammary gland. Prior to each imaging session, a low-resolution overview tilescan (top panel) can facilitate the identification of the region(s) of interest over subsequent imaging days. The collagen I pattern (second harmonic generation, magenta), tissue structure and patterns of differentially labeled cells with fluorescent proteins (depicted in green, yellow, and red) can be used to retrace the same tissue area. Scale bars represent 1 mm (overview tilescan) and 100 µm (bottom panels). (**C**) Schematic representation of the *R26-Confetti* reporter construct. Upon tamoxifen-induced Cre-recombination, the Confetti construct stochastically recombines, resulting in the expression of one of the four fluorophores (nGFP, YFP, RFP, or mCFP).

Figure 4: Multi-day IVM of pubertal branching morphogenesis. (**A**) Pubertal *R26-CreERT2; R26-mTmG* mice in age between 4–6 weeks were injected with a low dose of tamoxifen leading to Cre-mediated recombination of the *R26-mTmG* allele and were imaged on days 1, 3, and 5 to follow the dynamic changes in developing mammary gland. (**B**) Schematic representation of the *R26-mTmG* mouse construct, which in non-recombined conditions results in ubiquitous expression of membrane tdTomato (mT). Upon Cre-mediated recombination, mT is switched for membrane eGFP (mG) expression. (**C**) 3D rendering of an elongating and branching mammary duct over multiple days imaged through an R.MIW in an *R26-CreERT2; R26-mTmG* female mouse at 6 weeks of age. (**D**) Single Z-plane images of the branching tip (top panels) and the elongating branch (bottom panels). mT is depicted in red, and mG in cyan, scale bars represent 100 µm (A and B). Single cells in the stroma are highlighted by white asterisks. Note that signal intensity was manually increased to highlight the single cells in the stroma, leading to a slightly overexposed appearance of the mammary epithelial cells. (**E**) Representative images of the inguinal lymph node of an *R26-CreERT2; R26-mTmG* female mouse imaged through an R.MIW, showing an overview tile scan (left panel), and zoom images (right panels) of a single Z-plane (top) and a 3D rendering (bottom). Second-harmonic generation (collagen I) is shown in green, mT in red, and mG in cyan. Scale bars represent 500 µm (overview tilescan) and 100 µm (zoom images). Figure panels C and D are modified from Messal et al. [2].

Figure 5: Multi-day imaging of the adult mammary gland. 3D rendered images of a mammary ductal structure of an adult female *Cdh1-mCFP* (cyan, marking the luminal cells by Ecadherin expression) mouse over multiple consecutive days. The collagen I pattern (second harmonic generation, magenta) was used to retrace the same ROI, and the *Cdh1-mCFP* signal was used to

mark the ductal (luminal) cells. Note that visibility after day 3 was improved by the opening of the R.MIW lid and repositioning of the tissue. Scale bars represent 100 µm.

Figure 6: Longitudinal IVM of proliferative heterogeneity in the adult mammary gland using the KikGR mouse model. (**A**) KikGR mice were imaged at day 0 before and after exposure to violet light. The imaging sessions were repeated on days 2, 6, and 10 after conversion. (**B**) Schematic depiction of the hypothetical outcomes upon photo-conversion of Kik-GR mammary epithelial cells following proliferation (left panel) and no proliferation (right panel) as a function of time. (**C**) The same area of the mammary gland was imaged through an R.MIW over a period of 10 days. Smaller regions were photo-converted on day 0 and show a similar dilution rate of the Kikume red signal over time, indicating an equal turnover rate throughout the epithelium. (**D**) Zoom images (single Z-planes) of the indicated regions in panel C show proliferative heterogeneity at the cellular level 6 and 10 days after photo-conversion. Scale bars represent 100 µm (panel C) and 10 µm (panel D). (**E**) Quantification of the red/green ratio of randomly selected cells (*n* = 48 cells) in three photo-converted areas. A high red/green ratio is indicative of a low dilution rate and low-proliferative activity, whereas a low red/green ratio is indicative of a high dilution rate and proliferation. Figure panels C- E have been modified from Messal et al. [2].

DISCUSSION:

The R.MIW allows longitudinal imaging of the healthy and diseased mammary gland in the native, and minimally disrupted environment and permits repeated visualization of the mammary gland at diverse developmental stages. The R.MIW design allows the window to be opened at any point during the experiment. Long-term visual access to the tissue of interest may be hindered, for example, by the accumulation of cell debris on the coverslip. In such cases, the R.MIW can be opened before or immediately after an imaging session to allow cleaning of the entire visible tissue area and lid. The removable lid also allows manipulations of the tissue as well as local application of substances, such as specific inhibitors and therapies, labeling dyes, or specific cell types of interest.

This method overcomes the limitation of the previously described mammary skinflap procedures[4,7,8,13], which are limited to one imaging session, and can visualize processes such as branching morphogenesis (**Figure 5**), homeostatic tissue turnover (**Figure 6**), or tumor growth at a cellular level (**Figure 3B**). Yet, at the same time, the R.MIW permits local manipulation of the tissue in between imaging sessions, which comprises a great asset of the R.MIW compared to the previously published MIW[3,18] and all other imaging windows[20,22]. When opening the R.MIW it is important to maintain aseptic conditions at all times to prevent any source of infection. The ability to open the R.MIW in an aseptic environment allows optimizing the imaging conditions prior to every imaging session, which greatly improves long-term visual access to the tissue of interest. Specifically, in the mammary gland, which is embedded in an adipocyte-rich stroma, this is of great value. Moreover, the replaceable lid could uniquely enable the local administration of therapeutics, different cell types, labeling dyes, image-guided microdissection, or any other local manipulation of the tissue without the need to terminate the IVM experiment. For instance, to study tumor initiation, specific cancer cell populations could be injected directly in the ductal tree or stroma at a determined IVM time-point and precise mammary gland location, as long as this

ROI is accessible through the R.MIW ring. To study tumor progression, specific cancer cell populations (at a specific location, in a specific micro-environment, or with a specific behavior) could be photo-marked during the IVM session and subsequently microdissected using a fluorescent dissection microscope after the opening of the R.MIW. Isolated cells could be further processed for downstream analyses, such as (single-cell) mRNA sequencing. By using this approach, one could couple *in vivo* cell behavior to molecular expression profiles. The advantage of local drug administration enabled by the R.MIW allows the tissue to be imaged prior to and directly after treatment. The interval needed to remove the mouse from the imaging box and to subsequently perform local drug administration after opening the R.MIW lid can be performed in minutes, which allows the immediate phase capture of fast-acting drugs.

We show that IVM of the mammary gland through the R.MIW is compatible with many different fluorescent reporter mouse models. The adipose-rich environment is challenging to image, and therefore the use of bright fluorophores is recommended. However, as shown here, even less bright fluorophores such as mCFP can be visualized through the R.MIW in optimal imaging conditions. Inevitably, the fat pad will preclude the imaging of the deeper ductal structures, and limit the imaging to the more superficial ducts. A low-resolution overview image at the start of each IVM experiment will help to identify the ductal structures of interest that are sufficiently superficial for high-resolution imaging. Local tissue manipulation, removal of connective tissue, or repositioning of the adipose tissue after opening the R.MIW can optimize the IVM for specific ROIs that are overlaid by adipose tissue. This is an important advantage over all previous window designs, which do not allow to perform these manipulations and would require complete removal of the window itself. Specifically, for visualization of the inguinal lymph node, it is recommended to gently remove the overlying fatty tissue, which reduces light scattering and enables high-resolution imaging. When manipulating the tissue, always keep aseptic conditions and prevent bleeding or serious damage to the tissue, as they may affect the processes being studied during the IVM experiment.

The R.MIW is made of titanium, a material that is commonly used in clinical practice to replace hard tissues such as joints or bone plates. Titanium has several advantages over steel windows, including its lightweight and inert character[21]. Recently, several other materials were used to generate novel imaging window types, including the flexible silicon window[20]. In contrast to the R.MIW, the flexible window does not require any sutures for implantation and is suitable for nearly any anatomical position, specifically in the case of soft and fragile tissues. Silicon windows have a minimal impact on animal motility due to their lightweight and deformable nature and maybe more suited in experiments studying rapid tissue expansion and growth[20]. Another advantage over the titanium version is that silicon windows are compatible with other imaging modalities, including magnetic resonance imaging[20,27]. However, it will be important to keep in mind that objectives are optimized for 0.17 mm glass coverslips. Moreover, mammary tissue is susceptible to breathing movements, which are hard to restrict using the flexible window, especially when using an inverted microscope. Breathing artifacts are minimized by the R.MIW design and the fixation of the R.MIW in the inlay of the imaging box. As a result, images acquired using the proposed R.MIW setup are not distorted due to breathing artifacts. However, minor drifts in tissue localization can occur, which are usually gradual and can be corrected by using

post-acquisition motion correction software[28]. With the increasing toolbox of IVM technologies[2,20], the specific requirements for each experiment will eventually determine the best way of *in vivo* visualization of the tissue of interest. Different window designs have different advantages and disadvantages and depending on the research question, the available microscopy setup, the required spatial and temporal resolution, and the total time span of the studied process, the optimal approach needs to be determined.

In summary, the R.MIW facilitates high-resolution characterization of the cellular dynamics during mammary gland development, homeostasis, and disease over multiple days to weeks.

ACKNOWLEDGMENTS:

This work was supported by the Boehringer Ingelheim Foundation (PhD Fellowship to C.L.G.J.S), an EMBO postdoctoral fellowship (grant ALTF 1035-2020 to C.L.G.J.S.), and the Doctor Josef Steiner Award (to J.v.R).

DISCLOSURES:

The authors have nothing to disclose

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Fluorophore	Excitation wavelength (nm)	Detection range (nm)
mCFP	860	460-540
GFP	925	490-560
Kikume-Green	960	500-540
YFP	960	515-555
RFP	1040	560-640
tdTomato	1040	560-640
Kikume-Red	1100	580-620

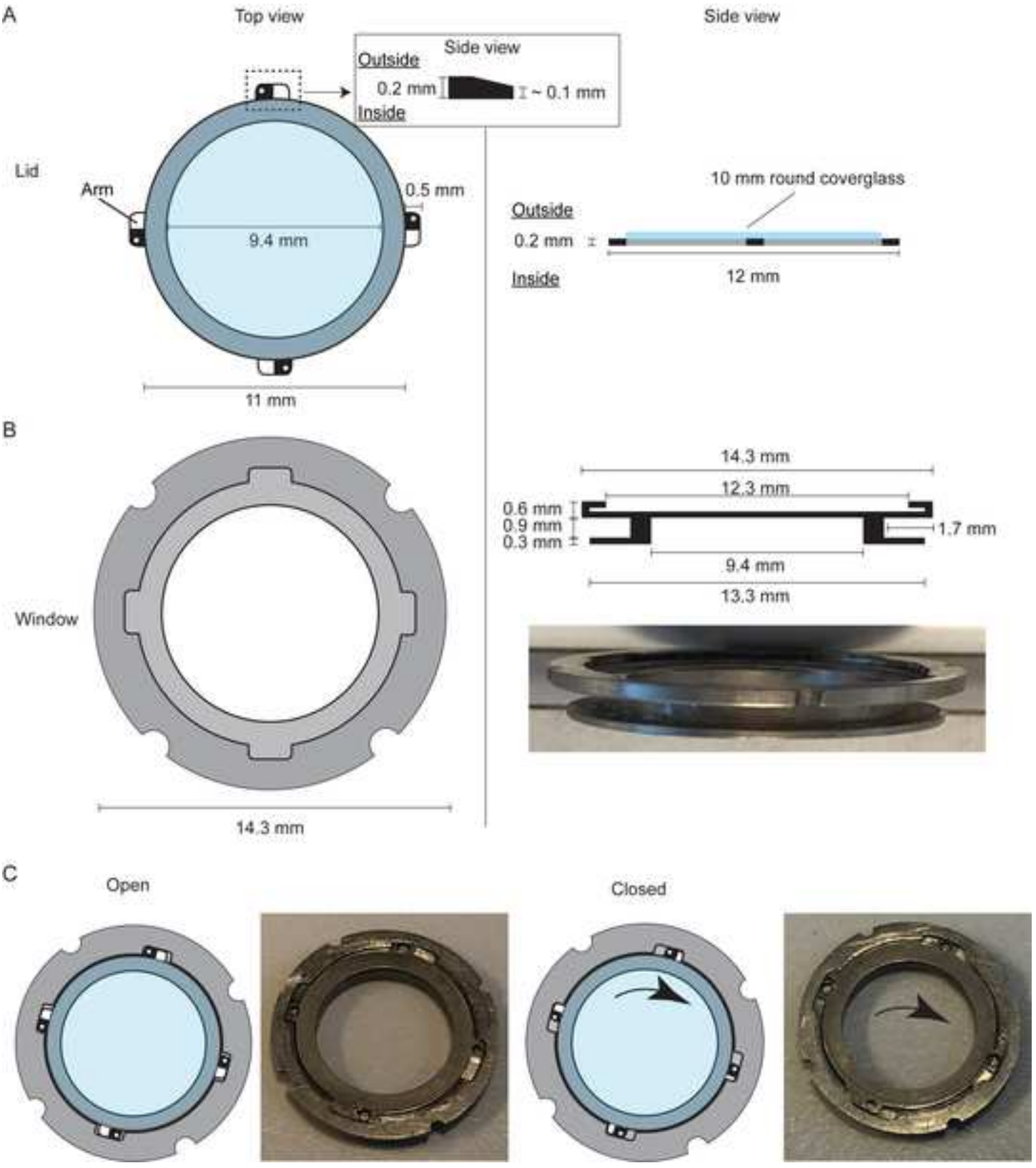


Figure2

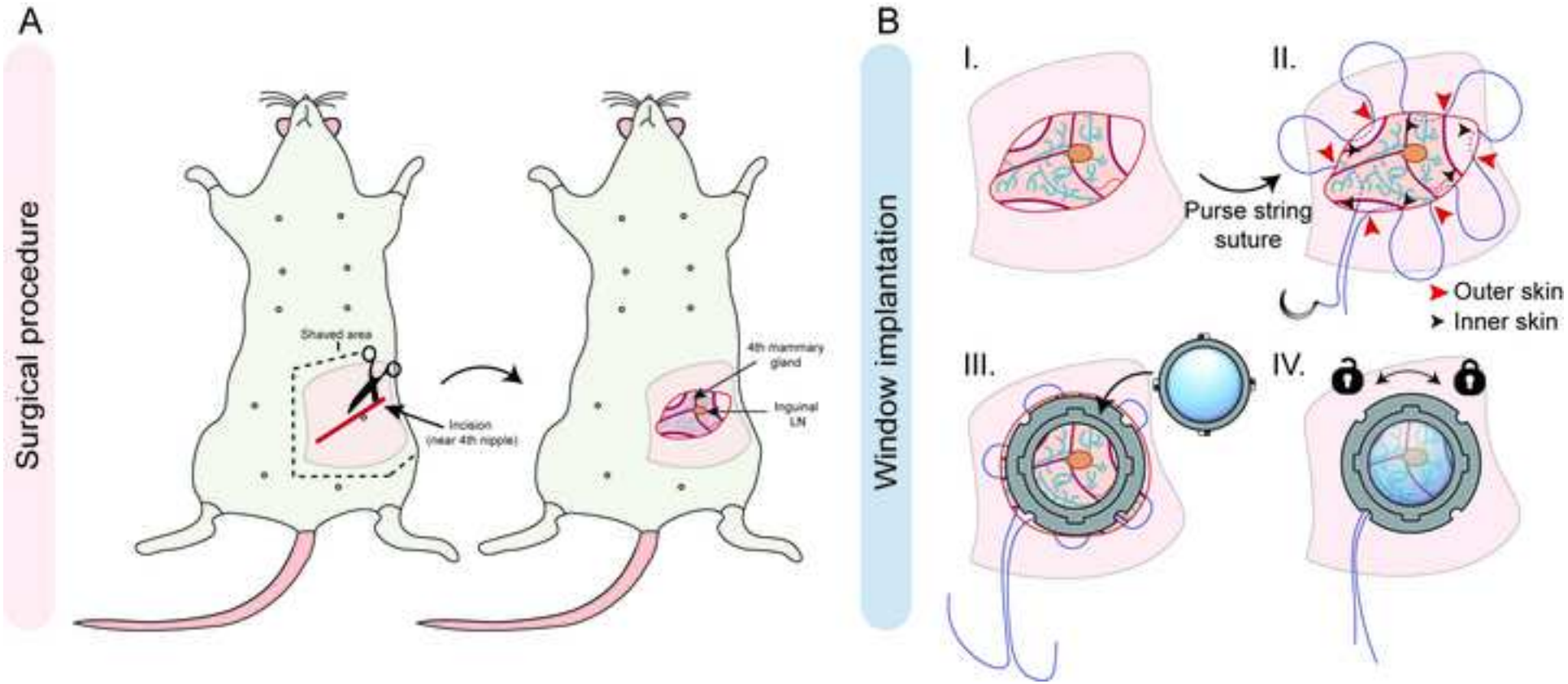
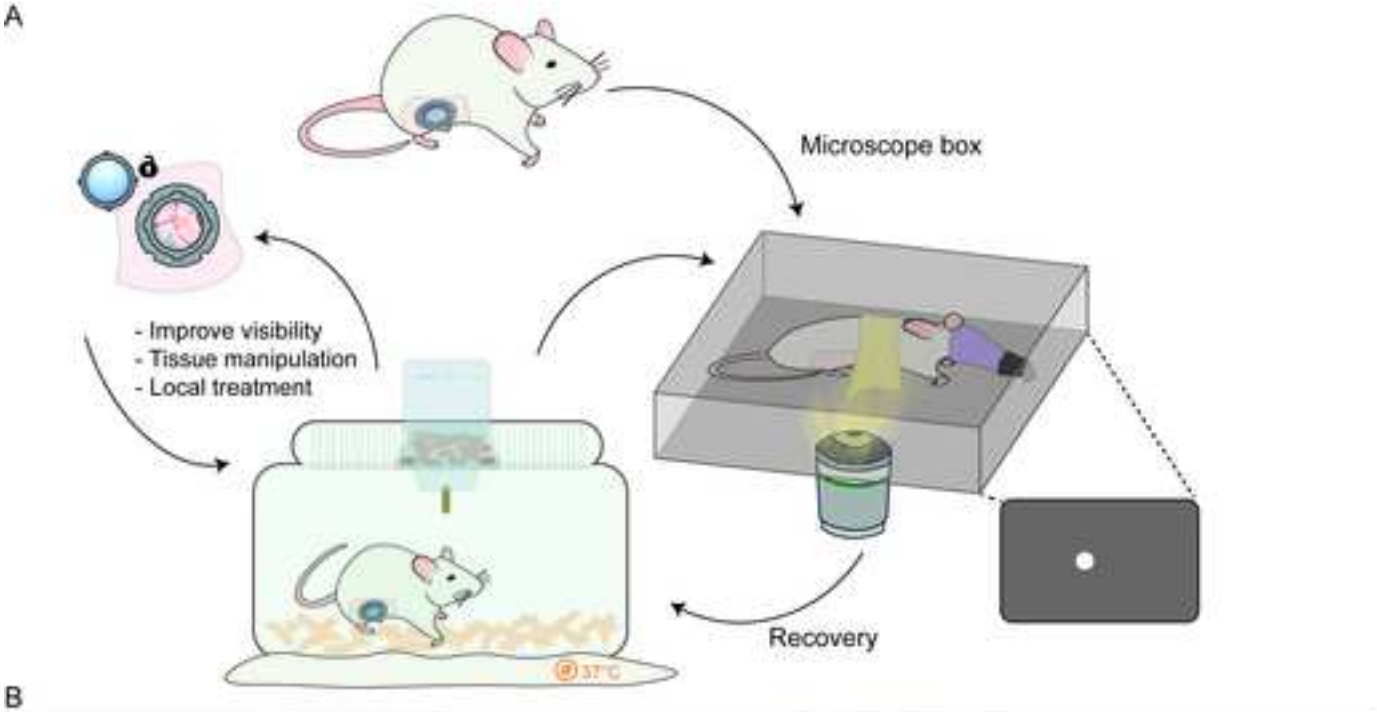
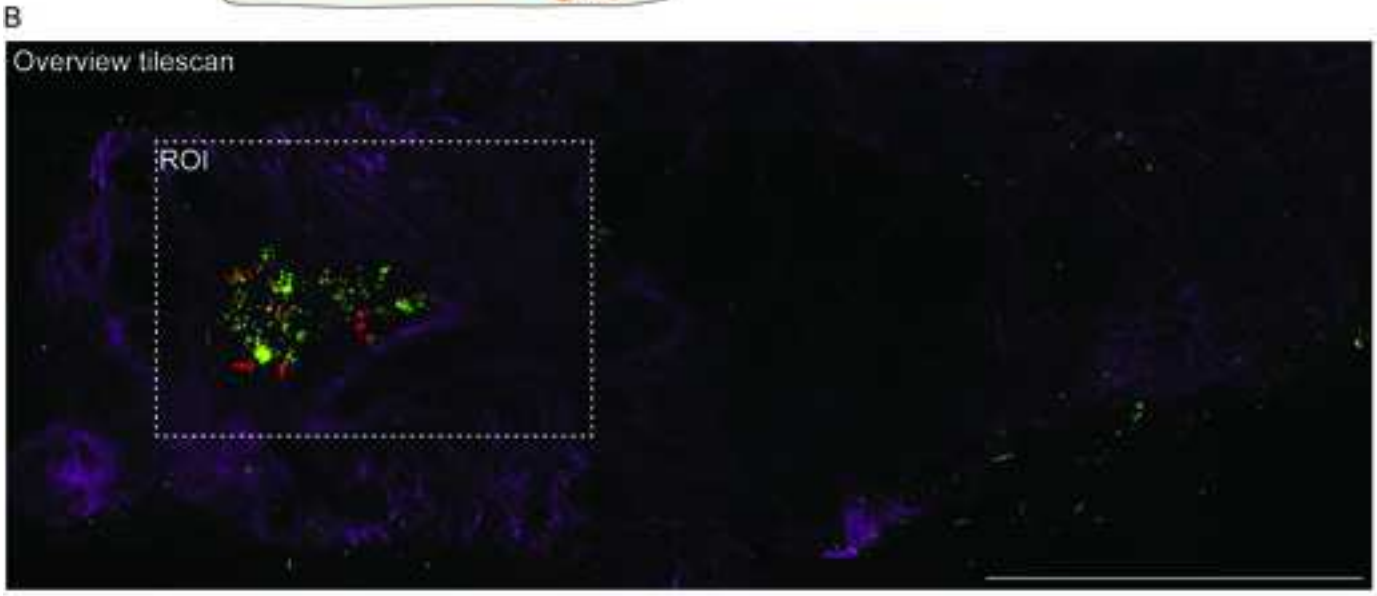
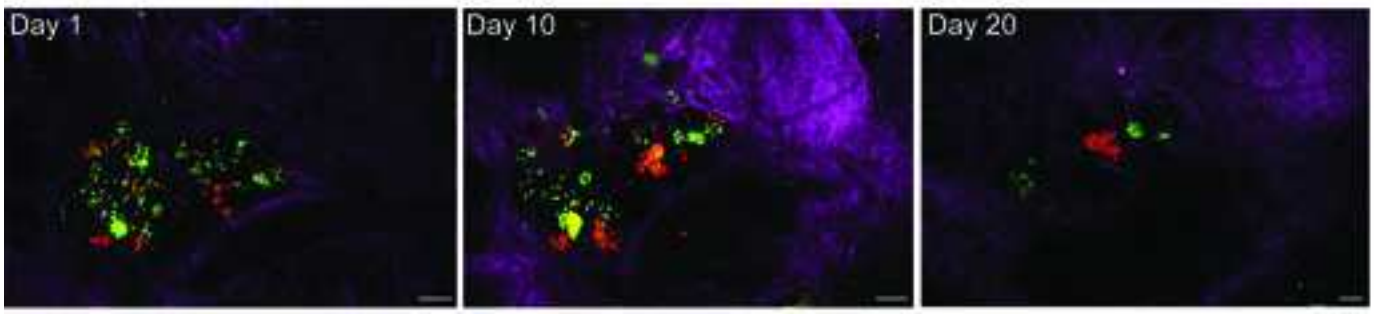


Figure3







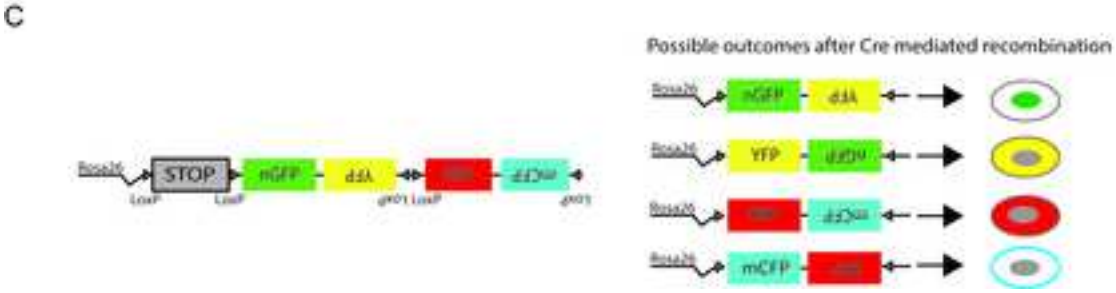
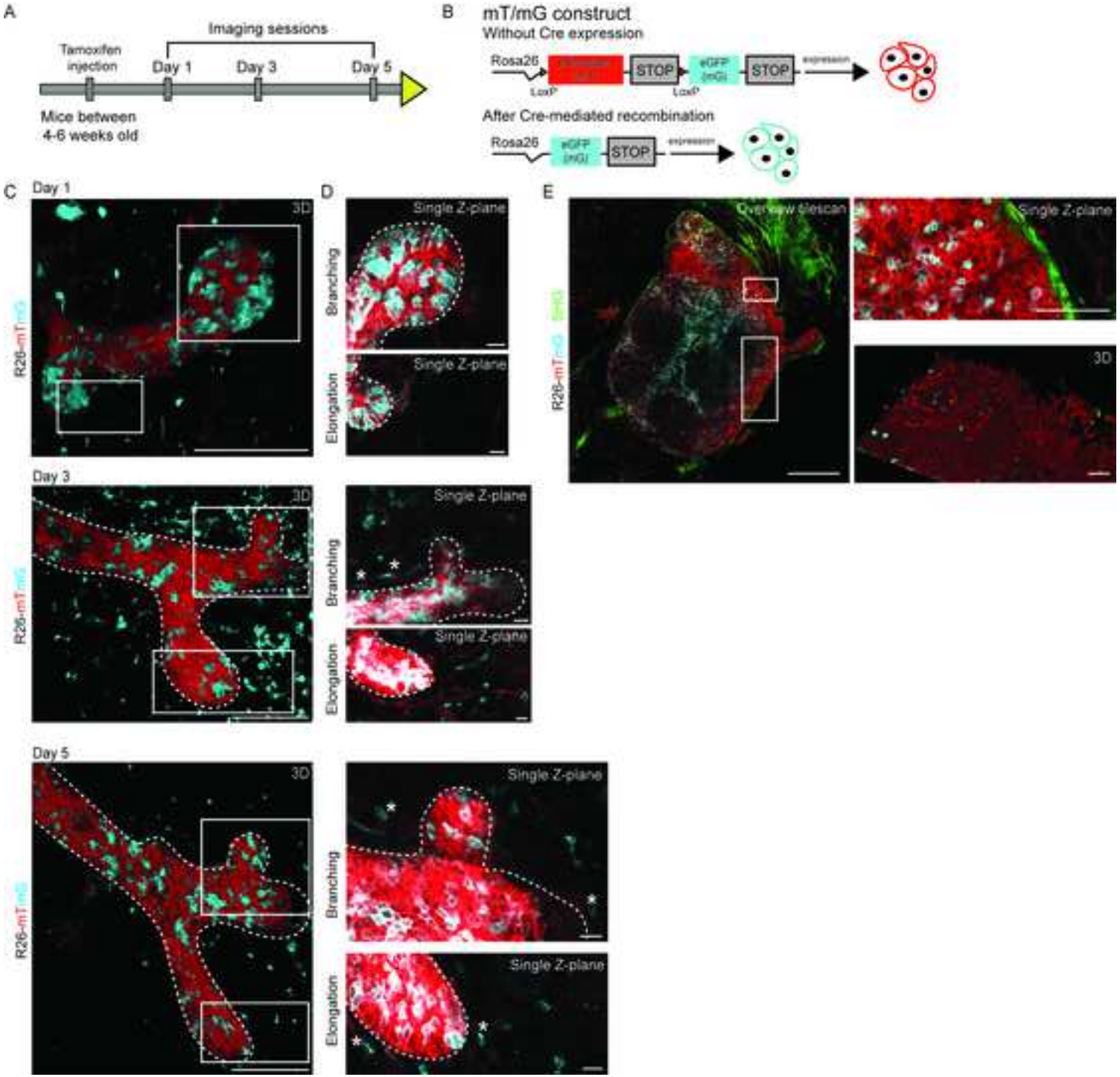


Figure4



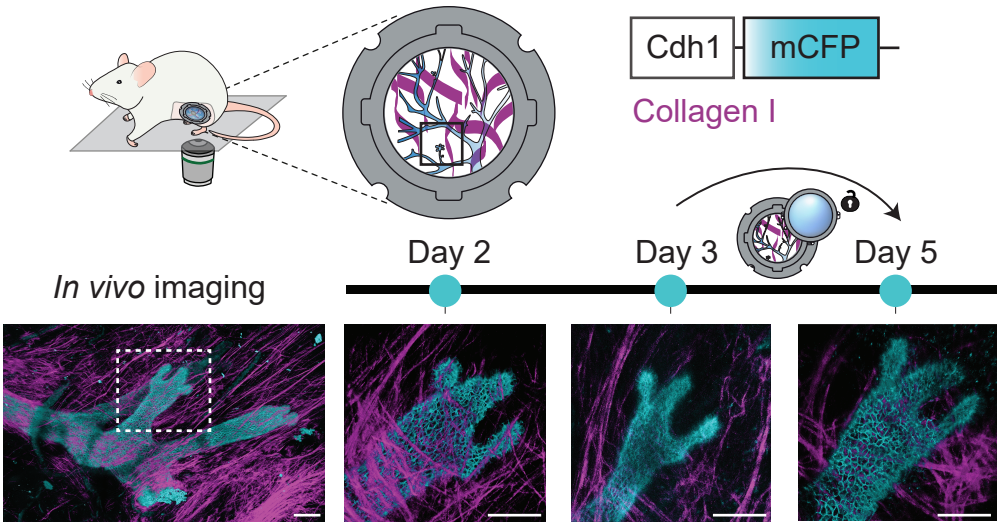


Figure6

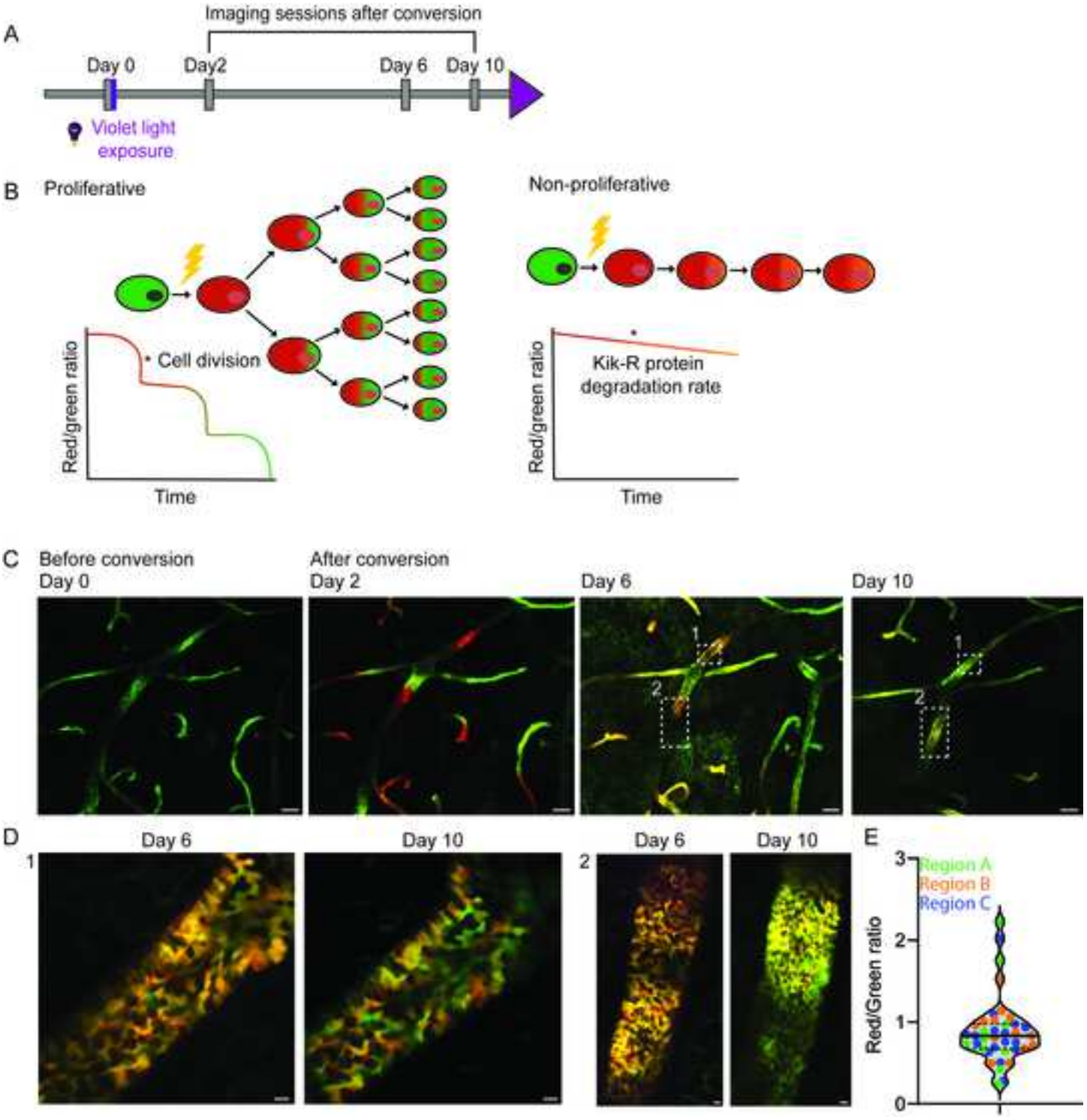


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Table of Materials

Materials_List_Final_RE.xls

Editorial comments:

Changes to be made by the Author(s):

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.

2. Please provide an email address for each author.

Emails have been added for each author.

3. Please revise the following lines to avoid overlap with previously published work: 156-157, 164-165, 185, 277=278, 299-300.

We have provided an alternative to each of the sentences. Now 232-233, 240-241, 283, 486-487, 515-516.

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

We have omitted the use of personal pronouns throughout the text to the best of our capacity.

5. 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 ensure the inclusion of 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 have added as many details as possible.

2.1: Please provide details of sterilizing tools etc.

We have provided the sterilization details in the manuscript. We will also stress this step within the video production.

2.3, 2.4, 4, 5: please specify how proper anesthetization is confirmed.

We have added the gold-standard method for anesthetization confirmation (paw withdrawal reflex) within the manuscript. We have also stressed that this step should be repeated during the critical time-points prior and during surgery.

3.11: Do you use any analgesics post-surgery?

We included the adapted analgesia (Buprenorphine) in the manuscript, including details on preparation and administration time-point for this surgical protocol.

4.7: please specify the microscope settings used here. Provide details of visualization by second harmonic generation; alternatively, cite a previously published protocol for the same.

We have added all imaging settings we use in the "results" section. The specific excitation/emission settings for 2-photon imaging are included a table (Table 1).

Line 270: please specify what kind of aseptic environment is needed and how it can be prepared.

In 2.1. and 2.2. we incorporated a detailed description of our sterilization procedure and surgical setup to ensure an aseptic environment before, during and after surgery.

What happened to the mice after the study? Did you euthanize them? If so, please specify the method without highlighting it.

We have added the euthanasia method utilized in our laboratory, by adding the following lines: "At the end of the desired experimental period, animals with an implanted R.MIW can be euthanized using a deep anesthesia with isoflurane followed by cervical dislocation. If desired, the organs of interest can be dissected for further ex vivo analyses" in section 4.11. Moreover, we elaborate on the possibility to keep the animals alive after the *in vivo* imaging experiment has ended in section 4.11.

6. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points and one-inch margins on all the side. Please include a ONE LINE SPACE between each protocol step and then HIGHLIGHT up to 3 pages of protocol text for inclusion in the protocol section of the video.

We adhered to the requested formatting to the best of our capacity.

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Editorial policies of the Journal of Mammary Gland Biology and Neoplasia is described here: https://www.springer.com/journal/10911/open-access-publishing#Fees%20and%20Funding

8. Figure 4: Scale bars must be given for all the panels in the figure obtained by microscopy.

Thank you for pointing it out. We have added the scale bar in Figure 4.

9. As we are a methods journal, please ensure that the Discussion explicitly covers the following with citations:
a) CRITICAL STEPS within the protocol

b) Any modifications and troubleshooting of the technique

c) Any LIMITATIONS of the technique

10. 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 6 and more than 6 authors, list only the first author then et al. Please include volume and issue numbers for all references, and do not abbreviate the journal names. Make sure all references have page numbers or if early online publication, include doi.

We have adhered to the required bibliographical format and included all necessary doi strings for early online publications.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This is a well-written manuscript describing a novel approach/protocol for performing intravital microscopy imaging of mouse mammary gland longitudinally for over a longer period of time. This intravital imaging approach is enabled by a novel imaging window with replaceable lid (R.MIW). A particularly useful feature of this replaceable lid is that it allows local tissue manipulation, local substance administration, or even image-guided microdissection. This approach should be very useful for people studying mammary gland development and mammary tumorigenesis.

Minor Concerns:
I only have several minor concerns/questions:
1. Since the replaceable lid is fitted with a thin glass coverslip, there is a concern of potential damage to the coverslip during mouse movement, which might generate an equivalent of "open wound" on the mouse skin.

If the coverslip is properly placed (as described in 1.2 and 1.3), mouse movements are not sufficient to damage the coverslip. Furthermore, one of the advantages of this novel window design is its replaceable lid, which allows for a quick replacement of the lid and/or coverslip in case of damage. We have included this point in Step 4.1.

2. In the Abstract, the authors mentioned that this approach "allows high-resolution imaging of the mammary gland up to several weeks". If the R.MIW is implanted in young mice (e.g., 3.5-5 weeks of age) and if the imaging experiment would be performed for up to several weeks, would there be any concern of the "stability" of the implanted R.MIW when the mouse is still growing in size?

The pouch suture used for R.MIW implantation ensures enough flexibility for the skin to adapt as the mouse grows. In this way, the window can be implanted in young mice without any concerns about stability. However, skin turnover in general can cause instability of the sutures, with the risk of the window frame becoming loosened. Too avoid this, the condition of the R.MIW should be inspected

prior to each imaging session (as described in step 4.1), and adjustments should be made where necessary. We now describe the limitation of suture stability in the discussion, lines 413 - 417

3. In Figure 4, it may help the readers understand the genetic labeling experiment better by including schematic diagrams for Cre-mediated conversion of mT to mG and for the timeline of tamoxifen injection and imaging.

Thank you for this suggestion. In Figure 4B we have included a scheme illustrating the mT to mG reporter construct conversion upon Cre activity.

4. Similarly, in Figure 6, it may be helpful to also include schematic diagrams explaining the G/R ratio and timeline of the experiment.

Thank you for this subsequent suggestion. In Figure 6A we have included an experimental timeline and in Figure 6B we have added a schematic explaining how the G/R ratio reflects cell proliferation.

Reviewer #2:
Manuscript Summary:

In this protocol, Mourao et al describe an improved titanium mammary intravital imaging window (R.MIW) for longitudinal, high-resolution intravital microscopy of the murine mammary gland. This window encompasses a removable lid that allows access to the underlying tissue, facilitating improved visibility and local tissue manipulation during the entire imaging timeframe. The authors describe the design and implantation of the R.MIW, providing a detailed protocol supported with clear diagrams. In addition, they provide examples of its utility for observing dynamic cellular and tissue level changes during different developmental phases of the mouse mammary gland, or in mammary tumors. Overall, the manuscript is well written, and I have no major concerns or requests.

Some specific, minor points to be addressed are provided below:

Minor concerns:

1) On lines 67-69 the authors state that the skin-flap technique is limited to a one-time experiment and does not allow longitudinal tracking over days or weeks. They also state this in the discussion (lines 368-369). However, they used the skin-flap technique to perform repetitive long-term imaging of adult mammary gland homeostasis in their related paper (Messal et al, Ref.2). Can the authors clarify/comment as to why they did not refer to this here?

The repeated skinflap is indeed possible and allows repetitive imaging of the mammary gland. However, this technique can only be performed at a low frequency, i.e. at maximum once a week, and can only be repeated a limited amount of times (until now we have only performed a skinflap three times on the same mouse). Therefore, the repeated skinflap is mostly suited to study large-scale remodeling of the mammary gland at longer timescales, and not well-suited to study cellular dynamics, which occur at much shorter timescales.

To clarify this point, we have now included the following lines: *"In contrast, the skinflap technique does allow for optimization and repositioning of the tissue during the imaging session and a skinflap can be repeated multiple times2. However, repeated imaging sessions through a skinflap are only possible when sufficient time (at least 7 days) is allocated in between surgeries to allow recovery of the skin, and is therefore mostly suited to study processes at longer time scales. Moreover, it is advisable to not perform this procedure many times because of its invasive nature and large risk of infections and scarring upon wound closure (lines 70 – 76)".*

2) Line 135 (step 2.7) - the use of ethanol to disinfect the surgical field is discouraged in many institutions. An alternative should be included (e.g. povidone-iodine (Betadine)).

Thank you for this suggestion. We were not aware of these institutional differences and have now included povidone-iodine as an alternative.

3) Lines 201-202 (Step 4.5.2.1) - a supplier name and reference for this commercially available sterile infuse mixture should be included in the table of materials?

We have included this mixture under the name "Infuse nutrients mixture" in the table of materials.

4) Lines 262-263 - can the authors provide an indication as to the average incidence of complications such as skin inflammation/necrosis/breakdown that precludes window maintenance?

We have extended this paragraph by adding the following lines: "*The R.MIW ring is made of titanium, one of the most biocompatible materials that does not promote inflammation or necrosis after implantation21. If the aseptic conditions are followed and the sutures are well executed, the R.MIW mammary gland implantation poses a low risk of postoperative complications. Moreover, unlike abdominal imaging window implantation[2], R.MIW implantation is not a limiting factor for mouse survival because the mammary gland is not a vital organ and allows daily imaging up to the limit specified in the ethical protocol. The only factor that limits the duration of window implantation is the homeostatic turnover of the skin, which will eventually lead to the sutures falling out after 4 - 6 weeks. Therefore, it is important to regularly have a close look at the stability of the sutures. If desired, the sutures can be removed and replaced by a new purse string suture under aseptic conditions (as described in steps 3.5 – 3.8) to reassure window stability (lines 402 - 413)".*

5) Lines 270-271 - when the authors refer to tissue repositioning, I assume they use sterile forceps to gently move (or raise) the gland? Or another tool? Similarly, in the discussion (lines 386 - 390) the authors refer to removing the connective tissue and overlaying fatty tissues. Given these types of manipulations can lead to damage-induced inflammation that may have unwanted effects on the processes under investigation, it would be advisable for the authors to demonstrate some of these manipulations when filming the window replacement steps (steps 5.4 and 5.7 highlighted in yellow).

To manipulate/reposition the tissue, one can use blunt tip forceps and gentle manipulation by hand to lift the mammary gland. Step 5.6 will be exemplified in the filmed protocol.

6) Figure 2 - the distinction between inner and outer skin arrows could be made clearer in panel B

The arrows are now colored in contrasting colors for better visualization of inner and outer suture points. Inner sutures lines were also modified from full to dashed in order to highlight their interior position within the tissue. In addition, we will highlight these steps in the filmed protocol.

7) Figures 4 and 5 - the authors' earlier work (Messal et al (Ref.2) where the figures have been reproduced from) should be cited in the text when referring to these figures e.g. for Figure 4 in Lines 279-283, and in Lines 299-305 for Figure 5.

We have added the references to the modified figures originated from our previous published work in the respective figure legends.

8) The images included in Figures 3 - 6 are of an outstanding quality. It would be helpful to include further details as to the microscope settings used for each of the models (e.g. recommended laser wavelengths) in their respective figure legends to assist those using similar models. Related to this, further details with regards to the reporter model used in Figure 3 should be included (R26-Confetti reporter?).

Thank you for these suggestions. We have included the microscope model and respective settings used for image acquisition (section 4.7). Optimal laser wavelengths have been included in each figure legend, and we have included a table (Table 1) listing the used wavelengths to excite the different fluorophores, as well as the detection range.

We have incorporated a schematic depicting the R26-Confetti mouse in Figure 3, and the conversion of mT to mG upon Cre recombinase activity in Figure 4.

9) Table of materials - Custom made titanium lid and mammary imaging window. If the authors outsourced the manufacture of these to an external company it should be named here as future users may want to engage with a company already familiar with manufacturing these windows.

Indeed, we have outsourced the manufacturing of the R.MIW in the past. Unfortunately, the company does not exist any longer. In fact, we are currently searching for a new manufacturer of the R.MIW. To emphasize this point, we have added a general remark on the manufacturing of the R.MIW in lines 120 – 123.

Reviewer #3:
Manuscript Summary:

The manuscript by Mourao et al., details the generation, implantation and use of a mammary imaging window with replaceable lid for long-term examination of mammary gland development via intravital multiphoton imaging. The authors state this new imaging window can be used for a range of experiments e.g. labelling cells with dyes, substance administration, and micro-dissection. These functions are all extremely beneficial for the intravital imaging community, especially for those having difficulties visualising and imaging the fatty mammary gland. However, the authors only demonstrate the window's use for gland repositioning and single cell tracing. Additionally, it is unclear as to whether the windows can be aseptically opened within an imaging session to administer fast-acting dyes and substances. I have major and minor concerns, detailed below.

Major Concerns:

1. There are exaggerated claims of novelty. The authors mention throughout the manuscript that e.g. "Current mammary imaging technologies are limited to a time frame of hours to days" (line 31-32) and "visualisation of the healthy mammary gland structure remained limited to a few days" (line 58). Yet, a manuscript they explore further in their discussion (Jacquemin G et al., 2021, Science Advances) has shown that their mammary window can be left in the animal for 35 days of imaging. Additionally, the group themselves have published an extremely similar titanium imaging window (albeit without a replaceable lid) and were able to image liver, pancreas, kidney, spleen and small intestine for up to a month (Ritsma L et al., 2013, Nature Protocols). They have neglected to cite this paper in this manuscript, and it should be mentioned/discussed. So, this manuscript is not the first to do long-term imaging, and in the mammary gland. Furthermore, the authors detail that the titanium window described here can be left in the animal for 6 weeks - yet, all of their example experiments (Figures 3-6) do not image for a period of more than 20 days. I would like to see an example experiment that has been imaged over the course of the claimed 6 weeks.

We consider the new window design here proposed as innovative in the field of intravital imaging, not only because of its extended animal implantation duration, but most importantly, because of its replaceable lid. To date, a window with a replaceable lid that allows the *in situ* administration of drugs, cells and/or any injectables or topical solutions, has not been addressed by the scientific community.

In the discussion, we review in detail the flexible window design published by Jacquemin et al., 2021, including the differences between the flexible silicon window and the R.MIW. However, we agree that it may be better to include the complementary, but at the same time very different, flexible window design up front to make the reader aware of alternative approaches. Therefore, we have added a reference to this paper in the introduction, lines 61 - 63.

The abdominal imaging window is specifically designed to visualize abdominal organs and is not suited to implant on top of the mammary gland (the window is too thick, and the groove for the skin is too wide). Therefore, we consider this paper irrelevant to mention in the introduction. However, we have now cited Ritsma et al. in the representative results (line 411) and discussion (line 553) and highlighted once more the advantages of our new model in comparison to the previous design.

The R.MIW design indeed allows for imaging up to 6 weeks after implantation. However, these data are included in a manuscript that is currently under revision. Hence, we cannot include these data in this manuscript. We have modified this claim as follows: *"If no complications occur, the R.MIW will allow for repeated intravital imaging sessions over several weeks post-implantation (lines 254 - 255)".*

2. Duplication from a recently published manuscript by similar authors (Messal HA et al., 2021, J Mammary Gland Biol Neoplasia). It is concerning that Figures 1, 4 and 6 appear to be duplicated from the supplementary of this paper, along with a similar protocol for implantation and imaging. It is common and valuable to release a protocol paper such as the one proposed here after an experimental paper, but is rather unorthodox to publish back-to-back techniques papers. The authors should specifically mention and discuss this manuscript early in their introduction, and clearly state why this proposed manuscript is different and worthy of independent publication.

First, we would like to emphasize that the nature of a JoVE protocol is to publish a visual demonstration of an existing protocol, alongside a detailed technical description, to allow the readers to reproduce

the procedure. In particular, for complicated procedures, such as the implantation of an imaging window and subsequent intravital imaging, the limited materials and methods sections of standard paper formats do not allow the inclusion of sufficient detail, much less a visual demonstration, to easily implement similar procedures in other laboratories. Motivated by the many requests for demonstrations that we received over the past years for the (R.)MIW implantation procedure, we opted for this JoVE protocol to help our peers. As such, we are disappointed to hear that our efforts are now considered as unorthodox.

Second, the purpose of our previous manuscript (Messal et al., 2021) was to summarize all *in vivo* imaging tools that we developed over the past years to track the dynamics of mammary tissue morphogenesis, including biological results. This work also included a general description of the R.MIW implantation procedure in the supplement. However, due to the nature of this manuscript, we did not provide any visual details on the procedure, which is clearly the aim of the current manuscript (as it is in the Journal of Visualized Experiments). We believe that the video that will accompany this manuscript will be of great value to assist the community lacking expertise in the intravital imaging field.

In this revision, we have added the references to the modified figures originated from our previous published work in the respective figure legends, which were published under the Creative Commons License 4.0 (https://creativecommons.org/licenses/by/4.0/), allowing to *"copy and redistribute the material in any medium or format".*

Taking aforementioned points into account, we feel that it is superfluous to state in a JoVE manuscript why an additional video recording of the procedure would be an added value to the community.

Minor Concerns:

1. The authors mention that the window can provide tissue access for e.g. "local manipulation, labelling, drug-administration or image guided micro-dissection" (lines 38-39), yet throughout Figures 3-6 have only demonstrated its use for tissue manipulation of models whereby single cells are labelled without the need to remove the window. The authors should therefore state that the window COULD be used to add fluorescent dyes and substances/drugs or opened for micro-dissection. Successful examples of fluorescent dyes should be provided. Additionally, as the authors have not provided an example, they should define what "image guided micro-dissection" means.

We have changed the sentence and now mention that the window could be used to add fluorescent dyes etc.

We have included a few sentences on image-guided microdissection, and how this could be used in combination with IVM through the R.MIW in the discussion, lines 563 - 568.

2. Similar to minor concern point 1, the authors say in lines 78-79 that their window "enables the local substance administration, such as different cell types of interest…". Firstly, this sentence should be re-written to be made clearer. Secondly, please define what cell types of interest are being added to the tissue beneath the window? The ability to add cell types of interest is mentioned throughout the manuscript (e.g. line 232, 248, 377). If it is a means to study e.g. tumour initiation, this should be stated.

We have rephrased the sentences in lines 78-79 (now lines 85 - 90) for reader clarity: *"When opened under aseptic conditions, the R.MIW does not only allow local tissue manipulation to improve optical*

access, but it also enables local administration of substances, such as pathway inhibitors or agonists, injection of different cell types of interest, such as cancer cell or immune cell populations, or tissue labelling-dyes. The lid can be opened at any moment in between imaging sessions without causing any damage to the underlying tissue."

We have included in the discussion in lines 560 - 563 a sentence exemplifying the use of specific cell populations: *"For instance, to study tumor initiation, specific cancer cell populations could be injected directly in the ductal tree or stroma at a determined IVM time-point and precise mammary gland location, as long as this ROI is accessible through the R.MIW ring."*

3. "The lid can be opened in between imaging sessions at any moment during the imaging experiment" (lines 80-81) - please clarify what this sentence means. It is unclear throughout the whole manuscript whether the window can be opened during an imaging session, while maintaining aseptic conditions. If the window cannot be opened during an imaging session, what are the implications for capturing the immediate phase of a fast-acting drug response? This needs to be discussed as a limitation of the window in the discussion.

We apologize for this confusion. We have modified this sentence for clarification: *"The lid can be opened in between imaging sessions at any moment, without causing any damage to the underlying tissue (lines 88 - 90)."*

The R.MIW lid can be opened during an imaging session, and therefore the time necessary to capture an image after drug administration is relatively short and should not take longer than 5 minutes after drug administration. In the discussion we have now included a sentence about the timing required for fast-acting drug responses: *"The advantage of local drug administration enabled by the R.MIW allows the tissue to be imaged prior to and directly after treatment. The interval needed to remove the mouse from the imaging box and to subsequently perform local drug-administration after opening the R.MIW lid can be performed in minutes, which allows the immediate phase capture of fast-acting drugs. (lines 568 - 572)."*

4. Line 89 - the authors have not shown that their window can be used to examine mammary disease. It should be mentioned that it COULD be used for studying disease. Additionally in line 369, the authors mention that their window can visualise processes of tumour initiation, yet this has not been shown in the manuscript.

In Figure 3B we show the growth of a hyperplastic/tumorigenic region over 20 days, indicating that the R.MIW is suited to study mammary disease. To be more precise, we have changed line 550 and now state that the R.MIW is suited to study *"tumor growth at a cellular level"*.

5. The authors should discuss the ethical implications of window implantation and doing long-term imaging. As the purpose of this manuscript would be to share the technique amongst different countries and institutes, some of which may not be familiar with long-term, live imaging, the level of detail here would be unhelpful for an establishing lab. The authors do mention that the analgesia strategy should be discussed with the local veterinarian and administered as per institutional guidelines (lines 96-97), which is correct, however as a helpful example they should detail the pre- and post-operative analgesia they used in their institution i.e. what type of analgesia, the dose, timing, implications and side-effects. It is great that the authors have included the clinical markers to watch

out for post-surgery (lines 175-178), but should also detail what to look out for after the animal has been imaged and the risks associated with imaging for >1 hour or anaesthetizing twice a day.

We are thankful for this suggestion. We have incorporated our protocol for analgesia in the protocol (Section 3.11).

Moreover, we have provided more details on the monitoring of the mouse during and after each imaging session in section 4.9.

6. The Table of Materials should be mentioned within the protocol text, and early on. Additionally, the fact that the window is custom made should be mentioned early in the text.

The Table of Materials is now mentioned in the Protocol section in lines 117 - 118: "All the necessary materials, manufacturers and catalogue numbers are provided in the Table of Materials, supplemented with this protocol."

The custom-made origin of the R.MIW is re-enforced in the introduction in lines 81-84: "The R.MIW was custom designed to provide optimal tissue access, enabling direct tissue manipulation (…)."

7. Line 99 - please add that anaesthetised AND recovering mice should be placed on a heating pad after both surgery and imaging

We have rectified this sentence (now lines 114 – 115).

8. Line 103 - the location of the arms of the imaging lid should be defined, perhaps this can be labelled in Figure 1A

We have included the word "Arm" in Figure 1A.

9. There is no mention of Figure 1B and 1C within the text. Please modify protocol in section 1 to include a sentence on how the window fits into the frame, mentioning the 5 degree turn

We have included a reference to Figure 1B and 1C in the introduction. In addition, we now refer to Figure 1B and C in Section 3.

10. The note in lines 114-115 should be moved underneath section 1.4 (line 111)

Thank you for this remark. Note has been moved underneath section 1.4.

11. The authors mention that the 4th nipple should be used as a landmark for window insertion (lines 132-133). What implications does this have for lactation and involution studies, where anatomically the nipple plays an important role in gland development and changing morphology?

The nipple is used as a landmark/orientation point to decide where to make the incision. For example, during puberty, when the gland did not invade all parts of the fat pad yet, this can be important to determine the place of window insertion. This does not imply one should detach or remove the nipple during the procedure. When imaging pregnancy/lactation, it would be advised to place the R.MIW further away from the nipple to allow suckling of the pups.

12. Line 169 - please modify to say petroleum jelly

Done.

13. Can the authors please comment on the volume of air between the coverglass and tissue that is introduced every time the window is opened, and how this impacts imaging?

We now provide a more detailed description of the procedure of R.MIW closing in step 3.9 and refer to this description in step 5.7.

14. Section 4 of the protocol - please add that eye ointment should be administered after the animal is anaesthetised (prior to imaging)

We have added this sentence.

15. Lines 281-282 - the authors say that "the dynamics of stromal cells surrounding the elongating and branching ducts" can be visualised, is this correct? There are no stromal markers in Figure 4B. Perhaps the authors mean "single" instead of "stromal".

We have modified this sentence and now state that single cells in the stroma can be visualized (lines 440 - 444). We have added asterisks to the figure panels to highlight these single cells in the stroma.

16. Lines 304-305, "Together, these data reveal a remarkable local proliferative heterogeneity within the adult mammary gland, and at the same time, a global uniform turnover rate". Could the authors please clarify what they mean by the "global uniform turnover rate" and whether the model they've used is capable of supporting this conclusion?

We would like to stress that this manuscript is meant to highlight and explain the use of the R.MIW, and not to provide a full representation of biological data and a justification of the use of models. Hence, we meant to highlight typical data types that can be obtained through the use of a R.MIW in the “representative results” section. For further details and additional data on the local proliferative heterogeneity and global uniform turnover rate we would like to refer to the previously published manuscript by Messal *et al.,* Journal of Mammary Gland Biology and Neoplasia 26, 9–27 (2021).

17. Figure 4B. As a single z-plane image, I am unsure why we are seeing white cells when, according to the mTmG model used, the cells should either be cyan or red. Can the authors please clarify?

Due to the strong stability of the mT (tdTomato) fluorescent protein, some recombined cells can display, over a short period of time, both the original mT protein previously produced and the newly expressed mG (GFP) protein. Also, please note that signal intensity in the single Z-plane images was manually increased to better visualize the single cells in the stroma, now indicated with asterisks. We have added this note in the figure legend.

18. Figure 6. Figure 6A and B shows two regions of interest, yet the quantification in Fig 6C is from 3 regions. Can the authors clarify where these 3 regions have come from, and whether region 1 and 2 is the same as in Fig 6A and B?

The quantification in Figure 6C (now Figure 6E) is not derived from the same images as depicted in Figure 6A and B. To avoid confusion, we have changed the naming of the 3 regions into Region A – C. Moreover, to make figure 6D more intuitive and consistent with the explanatory cartoon in Figure 6B, we now depict the Red/Green ratio (instead of the Green/Red ratio).

19. Post-acquisition motion correction software should be cited in line 409 so that others can use e.g. Warren SC, et al., 2018, eLife

We added the reference.

Reviewer #4:
Manuscript Summary:

The method of inserting titanium windows is given logical order with good detail and examples of the experimental utility of this technique are compelling. Advantages over alternative methods are presented. There is good coverage of experimental variables, especially in the discussion section.

Major Concerns:

The comprehension level of the second paragraph of the "Representative Results" section could be improved. Additional details would increase clarity. Perhaps different wording, further explanation, or an example would strengthen technical comprehension. Consider rewriting to something like, "A major challenge when using this approach is retracing a region of interest (ROI) on successive days. ……Several tissue landmarks can be used to find the same area of interest, including….."

We have re-phrased and corrected this paragraph as suggested: *"A major challenge when using the multiday imaging approach is retracing a region of interest (ROI) on consecutive days. To this end, a quick overview scan can be included before selecting the ROI(s) (Figure 3B). Multiple tissue landmarks can be used to retrace the same region of the tissue, including the collagen network signal (visualized by second harmonics generation), tissue structure, as well as local patterns of cells differentially or stochastically labelled with dyes or fluorescent proteins (Figure 3B). In this representative example, an R.MIW was implanted onto a mammary tumor in an MMTV-PyMT;R26-CreERT2het;R26-Confettihet female mouse. Subsequently, stochastic recombination was induced by injection of 1.5 mg tamoxifen, resulting in recombination of the Confetti construct in some cells (Figure 3C). The recombined tumor cells were followed for a period of 20 days (Figure 3B, C). If imaging of the same region of the mammary tumor over consecutive days is precluded, the window can be opened in an aseptic environment (as described in step 5.1) to further clear up, and reposition the tissue to improve visibility and image acquisition (Figure 3A)."*

The titanium windows and lids required for this approach are custom materials. It would be useful if the authors could discuss the feasibility and difficulty of making/obtaining these items. This would give readers valuable direction and information regarding the potential of using this method.

We have included a comment in the general remarks with the advice on how to produce the R.MIW parts. Typically, workshops specialized in manufacturing of titanium prosthetics have the tools and expertise to produce the R.MIW parts.

Minor Concerns:

There are minor typos and grammatical issues that will need to be corrected.

We have corrected all typos and grammatical mistakes that we could detect in the manuscript. Thank you for this observation.

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For Figures 1, 4, and 6.

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