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TITLE:

Using computer-based image analysis to improve quantification of lung metastasis in the 4T1 breast cancer model

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SUMMARY

We describe a more consistent and expeditious method to quantify lung metastasis in the 4T1 breast cancer model by using Fiji-ImageJ.

ABSTRACT:

Breast cancer is a devastating malignancy, accounting for 40,000 female deaths and 30% of new female cancer diagnoses in the United States in 2019 alone. The leading cause of breast cancer related deaths is the metastatic burden. Therefore, preclinical models for breast cancer need to analyze metastatic burden to be clinically relevant. The 4T1 breast cancer model provides a spontaneously-metastasizing, quantifiable mouse model for stage IV human breast cancer. However, most 4T1 protocols quantify the metastatic burden by manually counting stained colonies on tissue culture plates. While this is sufficient for tissues with lower metastatic burden,

human error in manual counting causes inconsistent and variable results when plates are confluent and difficult to count. This method offers a computer-based solution to human counting error. Here, we evaluate the protocol using the lung, a highly metastatic tissue in the 4T1 model. Images of methylene blue-stained plates are acquired and uploaded for analysis in Fiji-ImageJ. Fiji-ImageJ then determines the percentage of the selected area of the image that is blue, representing the percentage of the plate with metastatic burden. This computer-based approach offers more consistent and expeditious results than manual counting or histopathological evaluation for highly metastatic tissues. The consistency of Fiji-ImageJ results depends on the quality of the image. Slight variations in results between images can occur, thus it is recommended that multiple images are taken and results averaged. Despite its minimal limitations, this method is an improvement to quantifying metastatic burden in the lung by offering consistent and rapid results.

INTRODUCTION:

One in eight women will be diagnosed with breast cancer in her lifetime, and yet despite multiple treatment options breast cancer is the second leading cause of cancer-related deaths in American women[1]. These women are not dying from the primary tumor in their breast. Instead, the metastatic burden is responsible for the mortality of this disease as it commonly spreads to the lung, bone, brain, liver, and lymph nodes[2]. Because of this, breast cancer models need to evaluate metastasis to contribute to curbing the mortality of this disease. The 4T1 murine breast cancer model is a superb protocol to accomplish this. The method described here offers an improvement to the 4T1 model by using Fiji-ImageJ to quantify lung metastasis, producing consistent and expeditious results.

The 4T1 model is well-established, with most labs using protocols such as those described by Pulaski and Ostrand-Rosenberg in 2001[3]. The 4T1 cell line is 6-Thioguanine (6TG) resistant and representative of stage IV, triple negative breast cancer[3-5]. It is clinically relevant as it is an orthotopic model and spontaneously metastasizes to the same organs as in human breast cancer[3,4]. The 4T1 cells spontaneously metastasize at a predictable rate based on the quantity of cells injected[3,4]. Importantly, genetic differences between mice used here caused expected inter-individual variability in metastatic burden. To evaluate metastasis, tissues are harvested to collect and quantify cancer cells in distant sites using 6TG selection and methylene blue staining. The result is a collection of tissue culture plates with blue dots representing metastatic colonies. However, the Pulaski and Ostrand-Rosenberg protocol quantifies metastatic colonies by manually counting them, and therefore this has been the standard means of evaluating metastasis in this model. While this is easy for tissues with low metastatic burden, tissues like the lungs are often laden with metastases. As lung plates can be highly confluent, accurately and precisely quantifying metastatic colonies by manual counting is difficult and prone to human error. To better quantify metastatic burden, we describe using Fiji-ImageJ for a computer-based solution to human counting error. Histopathological analysis with hematoxylin and eosin (H&E) staining is another means to quantify lung metastases, and interestingly has also been improved with Fiji-ImageJ software[6,7]. However, because histopathological analysis observes a single slice of the lung, it can be inaccurate and unrepresentative. This is because the 4T1 model causes several metastatic lesions throughout the organ that are not evenly distributed. While overall

trends between histopathological analysis and manual counting can be similar[8], individual values can differ and therefore histopathological analysis should not be used as the sole means of quantification. We demonstrate the benefit compared to histopathological analysis and the inconsistencies in manual counting between different counters, while also demonstrating the consistency of using Fiji-ImageJ. Additionally, we show that this method can reduce the incubation time from 10-14 days to 5 days, meaning researchers can analyze data from their study much sooner than when relying on manual counting.

This method is a collection of simple adjustments to the Pulaski and Ostrand-Rosenberg protocol[3]. Because the 4T1 model is widely used, and because lung metastasis is a critical parameter to measure in preclinical models, we believe this method can be widely used and is highly valuable to breast cancer researchers. The only additional supplies needed are a camera and access to a computer with Fiji-ImageJ, a free software used frequently in image analysis[9]. This method specifically focuses on lung metastasis, but it could be used for other tissues with significant metastatic burden.

PROTOCOL:

All methods described here have been approved by the Institutional Animal Care and Use Committee (IACUC) of Virginia Tech and in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Performing this protocol requires permission from the appropriate institutions and adherence to all appropriate guidelines.

1. Cell Culture

1.1. Make complete culture media (RPMI + 10% Fetal Bovine Serum +1% Pen Strep). Revive 4T1 cells according to ATCC Protocol[10] and incubate at 37 °C and 5% CO_2 in a T-25 flask until confluent. Change media the day after reviving to remove dead cells, and again if media is spent before cells are confluent enough to passage.

1.2. Once the T-25 flask is confluent, passage cells to a T-75 flask by discarding media, washing flask with 5 mL of 1x Dulbecco's Phosphate Buffered Saline (DPBS), and adding 500 µL of Trypsin-EDTA. Incubate for 5-10 minutes at 37 °C until cells detach.

1.2.1. Once detached, add 5 mL of warmed complete culture media to cells. Aspirate and transfer the 5 mL to a T-75 flask containing 15 mL of warmed complete culture media.

1.3. Passage cells in T-75 flasks at least four times. Do this once the flask is confluent by washing with 8 mL of 1x DPBS, adding 1 mL of Trypsin-EDTA for detaching cells, adding 10 mL of warmed media to cells, and diluting 1:6-1:8 into a new T-75 flask containing 20 mL of warmed complete culture media.

1.4. Passage cells up to the appropriate number of T-150 flasks containing 40 mL of warmed complete culture media for the number of mice to be injected. Most studies will require multiple

T-150 flasks to ensure enough cells for injection.

1.5. When mice are ready to be injected (8 weeks old or weighing over 20 g, depending on the IACUC or institutional protocols), harvest cells by discarding media, washing each flask with 10 mL of 1x DPBS and adding 2 mL of trypsin-EDTA. Incubate for 5-10 minutes at 37 °C until cells detach.

1.6. Wash flask with 10 mL of media and transfer all contents (10 mL of media + 2 mL of trypsin-EDTA cell mixture) to the next flask. Continue to wash and collect cells from each flask using the same 10 mL of media to avoid using an excessive amount of media.

1.6.1. Once all flasks have been collected, transfer the contents into a 50 mL centrifuge tube. Collect a 10 µL sample for counting in a microcentrifuge tube and centrifuge the 50 mL conical tube at 125 x *g* for 5 minutes.

1.7. While cells are being centrifuged, add 10 µL of Trypan blue to 10 µL of cell sample. Count cells using a hemocytometer. Once the total number of cells is determined, calculate the concentration of cells needed to inject mice for 1.2 x 10^6 cells per mouse (per 100 µL).

1.8. After centrifugation, decant media and resuspend cell pellet in correct amount of sterile 1x DPBS for 1.2 x 10^6 cells per 100 µL. Split cell/DPBS mixture into microcentrifuge tubes for easy access with the syringe when aspirating cells for injection. Keep cells on ice and inject soon thereafter as cells will begin to die after being on ice for extended periods of time.

2. Injections

2.1. Prepare cells for injection by tapping or gently mixing the microcentrifuge tube to resuspend the cells, and then aspirate 600 µL into a 1 mL syringe. Turn the syringe upwards and pull the plunger down to bring cells away from the syringe opening. Tap the syringe to rid it of air bubbles.

2.2. Attach the needle bevel up and dispense cells back into microcentrifuge tube until only 500 µL remain in the syringe. Put syringe flat on ice.

NOTE: 4T1 cells fall out of suspension quickly. Therefore, it is important to mix cells back into suspension by tapping frequently.

2.3. Anesthetize 8 week old/>20 g female BALB/c mouse using isoflurane or other approved anesthetic agent. Monitor the mouse's breathing to assess depth of anesthesia.

2.4. Once the mouse is properly anesthetized as indicated by lack of corneal reflex, place the mouse on its back. Using the thumb, pointer, and middle finger, gently hold down the mouse. Use the pointer and middle fingers to hold down the mouse's upper body and thumb for its rear left leg. Be gentle but firm.

2.5. With the bevel of the needle up, inject 100 µL of cells subcutaneously into the mouse's left abdominal mammary fat pad. Monitor for a good bleb and any leakage, and ensure the mouse wakes up and moves easily after injection.

2.5.1. Change needles between each mouse.

NOTE: Do not allow needle to enter the peritoneal cavity. This would cause the cancer to spread quickly and not be representative of the model. To ensure a subcutaneous injection, gently pull upwards on the needle when inserted in the left abdominal mammary fat pad. If the needle is easily lifted upwards, it is correctly positioned subcutaneously.

3. Monitoring

3.1. Monitor mice at least 3 times a week for weight, body condition score, tumor size, tumor condition, respiration, activity level, appearance, and movement. Once the tumor reaches 0.7-0.8 cm in diameter, begin to monitor daily.

3.1.1. Consider euthanasia when tumor size reaches 1.5 cm, or weight loss reaches 20%, or severe clinical decline in body condition score, tumor condition, respiration, activity level, appearance, or movement are observed based on institutional guidelines.

NOTE: Body condition score is crucial to monitor as body weight may increase as the tumor increases in size, negating body condition loss due to disease burden. Exact monitoring protocols will depend on the approved IACUC or institutional protocols.

4. Necropsy

4.1. Euthanize mice using CO_2 following institutional guidelines.

4.2. Spray mouse with 70% ethanol to disinfect. Make an incision up the ventral midline of the mouse to expose the body cavity.

4.3. Remove the kidney. Continue cutting up the midline until the diaphragm is visible. Use scissors to puncture the diaphragm to deflate the lungs. Trim the diaphragm to get better access to the cavity.

4.4. Use blunt scissors to cut up the center of the ribcage. Pin ribcage back to expose the lung and heart.

4.5. Perfuse the heart with 2 mL of non-sterile 1x DPBS by inserting a needle into the apex of the heart until it pools in the abdominal cavity where the kidney was removed.

4.6. To remove the heart and lungs, use blunt scissors to cut the esophagus and trachea directly above the heart. Using forceps, begin to pull the heart away from the body and cut away at any

connective tissue keeping it attached. The lungs will come out with the heart.

4.6. Identify the multi-lobed (right) and single-lobed (left) lungs. Keep heart attached for reference, but once lungs are identified, cut the heart away.

4.7. Label a 12 well plate containing 1x Hank's Balanced Saline Solution (HBSS) in each well. Each mouse needs 2 wells. Place the multi-lobed (right) lung in the 12 well plate for metastasis evaluation and keep on ice. Keep the neighboring well empty for now.

NOTE: It is important to use the same lung (multi-lobed) from every mouse to ensure each sample is close in size. The single-lobed lung can then be used for other analysis, like histopathology.

NOTE: Samples are stable on ice or at 4 °C for a few hours.

5. Processing Tissues

NOTE: All steps in this section should be done using sterile technique.

5.1. Label 1 15 mL conical tube per mouse and add 2.5 mL of type IV collagenase mixture and 30 units of elastase to each tube. To make type IV collagenase mixture, dissolve 2 mg of type IV collagenase per mL 1x HBSS and sterile filter. This can be stored up to 12 months at -20 °C and thawed when needed.

5.2. Transfer the lung to the second, clean 1x HBSS well for that sample. Swirl using forceps to remove any remaining blood. Transfer clean lung to empty 1 cm tissue culture plate. Mince lung with scissors. Rinse plate with 2.5 mL of 1x HBSS, transfer 1x HBSS and lung pieces into a 15 mL conical tube already containing collagenase/elastase cocktail (5 mL total).

5.3. Incubate for 75 minutes at 4 °C. Continue mixing samples during this time, so place tubes on a rocker or rotating wheel. During this incubation step, label 50 mL centrifuge tubes and 10 cm tissue culture plates for each mouse. If doing a dilution, label enough 10 cm tissue culture plates for the dilutions.

NOTE: Label the lid of the tissue culture plates. If labeling the plate itself, the writing will interfere with Fiji-ImageJ analysis.

5.4. Bring volume of each tube up to 10 mL total with 1x HBSS. Pour contents over a 70 µm cell strainer into a 50 mL conical tube for each sample. Use the plunger of a 1 mL syringe to gently grind the sample through the strainer to allow more cells to filter through.

5.5. Centrifuge for 5 minutes at 350 x *g* at room temperature (RT). Discard the supernatant and wash pellet with 10 mL of 1x HBSS. Repeat this step twice.

5.6. Resuspend pellet in 10 mL of 60 µM 6TG complete culture media, either RPMI or IMDM.

Plate samples in 10 cm cell culture plates, using a dilution scheme if desired. Incubate at 37 °C, 5% CO_2 for 5 days.

NOTE: 1:2, 1:10, and 1:100 are common dilutions that will need to be empirically determined based on study parameters.

CAUTION: 6TG is toxic. Use caution when handling and follow all Environmental Health and Safety guidelines for disposal.

6. Staining plates

6.1. Pour culture media off plates into appropriate waste container. Fix cells by adding 5 mL of undiluted methanol per plate and incubate for 5 minutes at RT, making sure to swirl methanol so that it covers the entire plate.

CAUTION: Methanol is hazardous if ingested, inhaled, or is on skin. Use a fume hood for this step.

6.2. Pour methanol off plates into appropriate waste container. Rinse plates with 5 mL of distilled water per plate and pour water into appropriate waste container. Add 5 mL of 0.003% methylene blue per plate and incubate for 5 minutes at RT, making sure to swirl methylene blue solution so that it covers the entire plate.

6.3. Pour methylene blue into appropriate waste container. Rinse plates again with 5 mL of distilled water per plate. Turn plates upside down and blot against a paper towel to remove excess liquid. Place plate on its lid and let air dry overnight at RT.

NOTE: Metastatic colonies will be blue. Once plates are dried, they can be stored at RT indefinitely.

7. Image analysis

7.1. Remove labeled lids from plates, taking care to ensure clear identification of samples. Line up all stained lung plates on a clean, light surface to take a picture of all of the plates in one image.

7.2. Take a picture of the collection of plates in a well-lit area, making sure to minimize reflections as the plates are very reflective. Reflections in the plates will influence image analysis and therefore need to be avoided.

NOTE: Fiji-ImageJ has an upper limit of 2 gigapixels. Most modern smart phones will have sufficient cameras. Do not use a camera less than 8 megapixels. The camera used in this experiment was a 12.2 megapixel on a Google Pixel 2.

7.3. Crop the image to include the plates, but exclude the lids or anything else in the background

of the image. Upload the cropped image into Fiji-ImageJ.

7.4. Change the image to black and white using the following commands: Image, Adjust, Color Threshold, Thresholding method: Default, Threshold color: B&W, Threshold space: Lab. Unselect the Dark background box. The image should now be black and white. Black represents the light background, and white represents the blue metastatic colonies.

7.5. Using the Circle tool on the Fiji-ImageJ toolbar, select the area to be analyzed. Draw one circle to use for all of the plates to ensure each plate is analyzed for the same-sized area. Choose a size that maximizes analyzed area on the plates while minimizing the background noise that appears on the edge of the plates. The size appears in the toolbar as it is drawn, so it is possible to make a perfect circle by monitoring the height and the width as the circle is drawn.

7.6. Analyze the selected circle to determine what percentage of the area is white, which represents the area of the plate that has blue metastatic colonies. Use the following commands: Analyze, Analyze Particles, Size ($pixel^2$): 0-Infinity, Circularity: 0.00-1.00, Show: Nothing, and check the Summarize box. Hit **OK**.

7.7. Record the % Area result. This is the percentage of the selected area that is white, and therefore represents the metastatic burden.

NOTE: It is recommended to either save the results in Fiji-ImageJ or copy/paste the entire results page into a separate document. If % Area results are unexpected or suspicious, it is then possible to see if any of the other measurements were also suspicious or if % Area was recorded incorrectly.

7.8. Move the circle, without altering its size by grabbing it in its center, to the next plate in the picture. Repeat steps 7.6 and 7.7 for all plates in the picture.

7.9. Repeat steps 7.1 – 7.8 on at least two more images. Once all plates and images have been analyzed, average the % Area results between different images for each plate to mitigate any inconsistencies between pictures.

REPRESENTATIVE RESULTS:

This method contains simple adjustments from the Pulaski and Ostrand-Rosenberg 4T1 protocol[3] and can be visualized in **Figure 1**. When 3 separate researchers manually counted metastatic colonies for 12 lung plates (1:10 dilution), the results were very inconsistent between different counters (**Figure 2A**). All researchers were directed to "count the metastatic colonies that appear as blue dots", yet the inconsistencies demonstrate the issue with manually counting highly-metastatic plates. The researchers had varying levels of experience with the 4T1 model. A board-certified veterinary pathologist analyzed H&E stained lung slides for metastasis as another method to compare to Fiji-ImageJ lung plate analysis (**Figure 2B**).

Using the Fiji-ImageJ analysis, 3 separate researchers analyzed 3 separate images of the collection

of 12 plates (1:2 dilution). Images were taken in two separate lab spaces with slightly different lighting. The arrangement of the plates or the angle from which the picture was taken were different between each image. In contrast to the manual counting results, the Fiji-ImageJ results were consistent between counters for each of the 3 images (**Figure 3A**). To determine if there were inconsistencies between the 3 images, the results from the 3 images and the 3 counters were combined per lung plate (**Figure 3B**). There are differences between images for some plates, but the overall trends are similar and it offers more consistency than manual counting. To account for the variations between the 3 different images, results from each image were averaged for each plate (**Figure 3C**). These averages provided consistent results between counters that accurately and precisely analyze metastatic burden. Therefore, this protocol suggests taking at least 3 images of the plate collection in different arrangements, from different angles, or in slightly different light settings, and then analyzing and averaging the results. The contrast between manual counting and Fiji-ImageJ analysis is visualized when comparing **Figure 2A** to **Figure 3C**.

Another way to demonstrate the improvements offered by this protocol is comparing the ranking of the plates from most to least metastatic burden between counters, based on the counts from **Figure 2** and **Figure 3**. Manual counting agreed on the most confluent plate, but all following ranks were inconsistent between counters (**Figure 4A**). Contrastingly, the ranks from Fiji-ImageJ analysis for each image were much more consistent between counters (**Figure 4B**). The consistency is also seen when results from each image for each plate were averaged (**Figure 4C**). We acknowledge that this protocol does not offer complete consistency between counters, but it is an improvement from manual counting when comparing **Figure 4A** to **Figure 4C**. Histopathological analysis differed from both manual and Fiji-ImageJ counting (**Figure 4D**).

To demonstrate the importance of avoiding reflections in the images, an image with a reflection of a hand and its subsequent Fiji-ImageJ analysis is shown (left) opposed to the same plate without a reflection (right) (**Figure 5A**). Other dark blemishes from a dirty background surface or blood sample residue on the plates can negatively impact Fiji-ImageJ analysis too. The blood plate in **Figure 5B** only has 2 metastatic colonies (noted by white arrows), but the dark residue (noted by black arrows) caused Fiji-ImageJ to consider it as 31.6% metastatic. Therefore, it is important to have a clean, light surface and to not use this method for blood samples as blood samples will typically leave residual dark spots on the plate that are not metastatic colonies.

FIGURE AND TABLE LEGENDS:

Figure 1: Protocol Schematic. This protocol focuses solely on analyzing lung metastasis in the 4T1 model. The general flow of this protocol includes growing 4T1 cells in culture, injecting BALB/c female mice with 4T1 cells in the left abdominal mammary fat pad, monitoring mice according to IACUC and institutional protocols, sacrificing mice and collecting the lung, collecting cells from the lung samples, plating and incubating cells in 6TG selection media, fixing and staining cells after 5 days, taking pictures of the plates, and analyzing using Fiji-ImageJ.

Figure 2: Manually counting metastatic cells and histopathological analysis have inconsistent results. A. 12 lung plates with a 1:10 dilution were manually counted by 3 separate researchers

instructed to count metastatic colonies the same way, although experience with the model varied between researchers. The number of metastatic colonies counted varied greatly between researchers. **B.** Histopathological analysis identified and quantified individual tumor cell aggregates, classified as metastases, present in H&E stained lung slides. High, medium, and low magnification images of one representative slide are shown.

Figure 3: Fiji-ImageJ analysis is accurate and precise in determining metastatic burden. A. 12 lung plates with a 1:2 dilution were analyzed by 3 separate researchers in 3 separate images of the 12 lung plates. **B.** Results from each of the 3 images by each of the 3 researchers were combined. **C.** Results from each lung plate from the 3 images were averaged. One-way ANOVA with Tukey's multiple comparison test determined no significant differences between counters for each lung plate. Data are shown as mean + SD.

Figure 4: Fiji-ImageJ analysis provides more consistent ranking of metastatic burden compared to manual counting and histopathological analysis. A. The same lung plates from Figure 2 were ranked from most to least metastatic based on the manual counts from Figure 2. **B.** The same 12 lung plates from Figure 3 were ranked from most to least metastatic based on the Fiji-ImageJ analysis from Figure 3A. **C.** The averages from Figure 3C were ranked from most to least metastatic. **D.** Lung slides were ranked from most to least metastatic based on histopathological evaluation.

Figure 5: Reflections and non-metastatic dark spots will negatively impact results. A. An image with a reflection of a hand taking the picture disrupts the Fiji-Image J analysis, as shown in comparing the reflection Fiji-ImageJ analysis (left) to the correct Fiji-ImageJ analysis (right) **B.** Blood plates often leave leftover stains (black arrows) on the plates that are not metastatic colonies (white arrows).

DISCUSSION:

As demonstrated, manually counting the metastatic colonies on each lung plate can be an inaccurate and imprecise method to quantify lung metastasis, demonstrating the need for a better means of quantification (**Figure 2**). Histopathological analysis differed slightly from both manual counting and Fiji-ImageJ analysis (**Figure 2B and 4D**), likely because the H&E slides are not a representative sample of the entire organ. The protocol harvests an entire lung, and therefore is more representative of total lung metastasis, and is more consistent than manual counting. Several different approaches to Fiji-ImageJ analysis were attempted and are discussed below, but the protocol outlined above appears to be the superior method.

Lung, blood, and brain samples were collected for this study. However, the blood and brain samples had very few metastatic colonies, if any at all. We determined that manually counting the metastatic colonies is optimal for these less-metastatic tissues, and therefore blood and brain data were not included. When the metastatic burden is easy to manually count (e.g., ten or twenty metastatic colonies as opposed to thousands), the original issue of human error is not relevant, and therefore this protocol is not needed. Also, blood samples can leave dark spots on the plates after fixation, which interferes with the Fiji-ImageJ analysis (**Figure 5**). Importantly, the

quantity of cells injected can influence the metastatic burden. For instance, if fewer cells are injected and the mice can survive longer, the cancer has more time to spread to the traditionally less-metastatic sites like the brain[3,4]. Therefore, this protocol could be modified to include the metastatic burden of other tissues if they are given time to become highly-metastatic. If trying the 4T1 model for the first time or changing the quantity of cells injected, we recommend trying at least two dilutions when plating cells. For this study, we used a 1:2 and 1:10 dilution. The 1:2 dilution would have been difficult to count manually, but was counted easily in Fiji-ImageJ. The 1:10 dilution was still difficult to count manually and therefore led to inconsistent results. Dilutions can be modified based on the specific study parameters.

Pictures were taken of individual lung plates and the 12 lung plates together. Individual plates were analyzed in two ways: either cropping the image to a central square of the plate prior to uploading to Fiji-ImageJ, or using the circle selection tool in Fiji-ImageJ to select the central circle of the plate in the uncropped image. We found that using the circle selection tool in Fiji-ImageJ offered the easiest, most consistent way to create a same-sized area for analysis for all plates. Furthermore, analyzing the entire collection of lung plates in the same image was superior to analyzing individual images of single lung plates. Having all of the lung plates in the same image allows for the same-sized circle to be used easily between the lung plates. It ensures all lung plates are the same distance from the camera and therefore the same-sized circle for analysis should be the correct size for all lung plates in the image. It also makes analysis quicker as redrawing the circle is not necessary between plates. It is simply dragged to the next plate in the image without changing its size, which guarantees the same size is used for all plates in the picture. When selecting the size of the circle, it is important to make it large enough to analyze the majority of the plate while small enough to avoid the background noise from the edges of the plate. Furthermore, in an attempt to save reagents, cells were also plated in 6 well plates and compared to the 10 cm tissue culture plates. The Fiji-ImageJ results from the 6 well plates were less consistent and did not correlate to the 10 cm dishes (data not shown). One explanation is the smaller surface area provides a smaller area to analyze, leading to less representative data. Another is that reducing the surface area allows the cells to grow more quickly as they are closer to other surviving cells. Therefore, we do not recommend using any tissue culture reagents other than what we have described in the protocol.

As mentioned before, avoiding reflections and having a clean, light background are absolutely critical to this method. **Figure 5A** demonstrates how a reflection is analyzed in Fiji-ImageJ and therefore shows the critical importance of avoiding reflections. As tissue culture plates are highly reflective, it is beneficial to take the picture at a slight angle to avoid reflections from either yourself taking the picture or from the light sources above. The lighting conditions of the specific work area will need to be accounted for. We suggest taking multiple pictures of the plates to be analyzed, trying slightly different arrangements and/or angles, in a well-lit area. Study the pictures intensely for any reflections. If there are inconsistencies in the analysis, it is likely due to a picture quality issue. To troubleshoot, compare the normal picture to the black and white picture. If areas that are not blue in the normal picture are appearing as white in the black and white picture, there is likely a reflection or blemish that is altering the results.

In addition to consistency, another notable benefit of this method is that it produces data much more quickly than manual counting. Manually counting multiple plates is very time-consuming, while Fiji-ImageJ analysis can be done quickly. It also allows for a shorter incubation time. Pulaski and Ostrand-Rosenberg recommend a 10-14 day incubation period for the plated cells, adding a substantial amount of time to the study[3]. The 10-14 day incubation period allows for larger, easier-to-count colonies to form. However, many lung plates can become confluent before then. Instead, 5 days of incubation gives enough time for the 6TG selection to kill non-cancerous cells (proven by healthy control mice not having any colonies on their lung plates, data not shown), and for the cells to grow enough to be easily quantified with Fiji-ImageJ. This significantly decreases the time between the mice being sacrificed and analyzing essential metastatic data.

To conclude, the benefits of this method far outweigh the limitations. We acknowledge this method does not offer perfect consistency. While this is not the ideal method for less-metastatic tissues, those tissues can easily be counted manually. While getting a picture without reflections can require some careful photography, the consistency gained with this method is significant. It is possible that this method could be used for other tissues that are highly-metastatic and other protocols that require counting stained objects. The study design could also allow for analyzing the rate of metastasis or effect of anti-cancer treatments on metastasis. This method will provide highly-consistent, reliable metastasis data and represents a significant refinement to the 4T1 model. The application of this model to upcoming breast cancer metastasis research is of utmost importance in arming researchers with tools to battle against breast cancer mortality.

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DISCLOSURES:

The authors have nothing to disclose.

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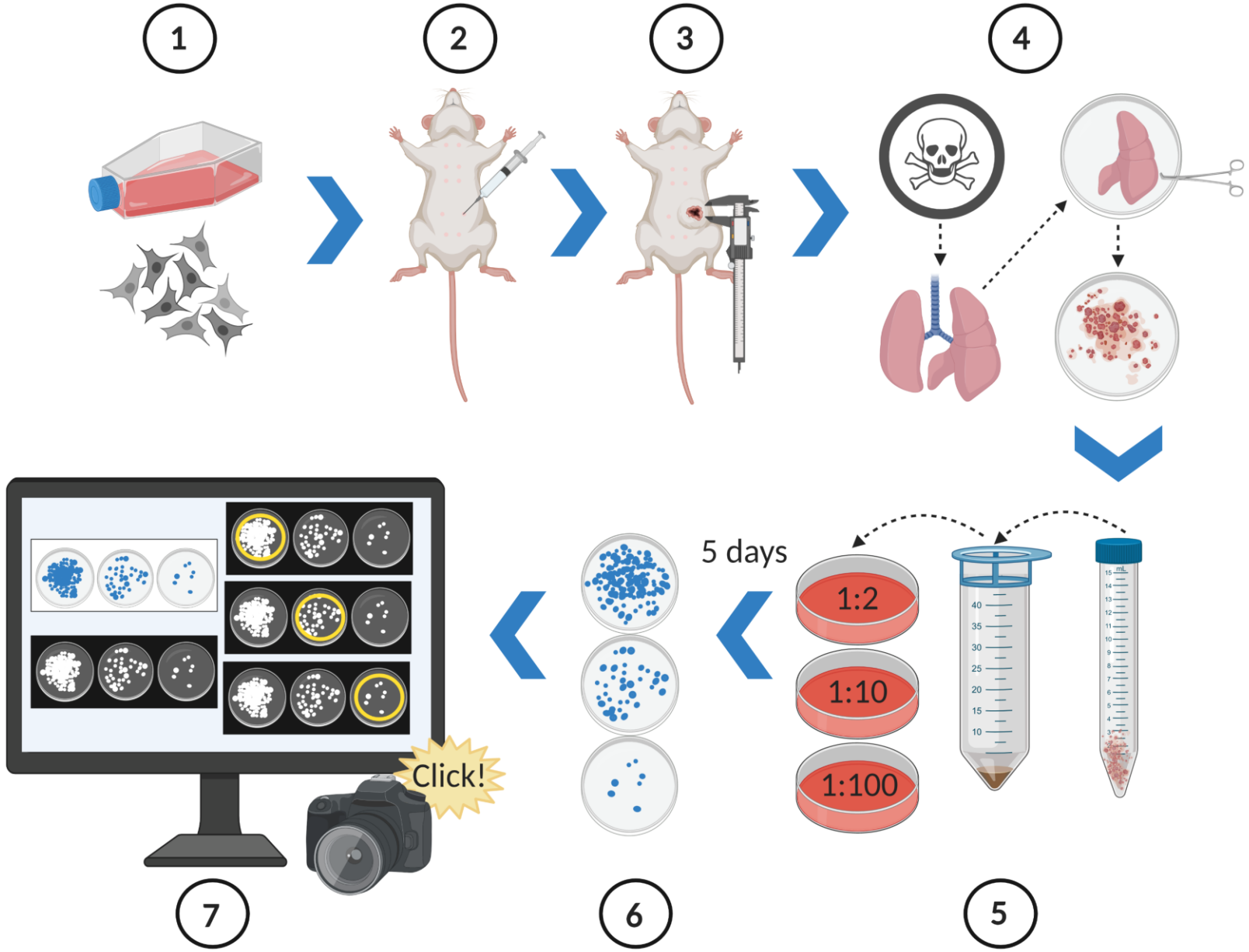
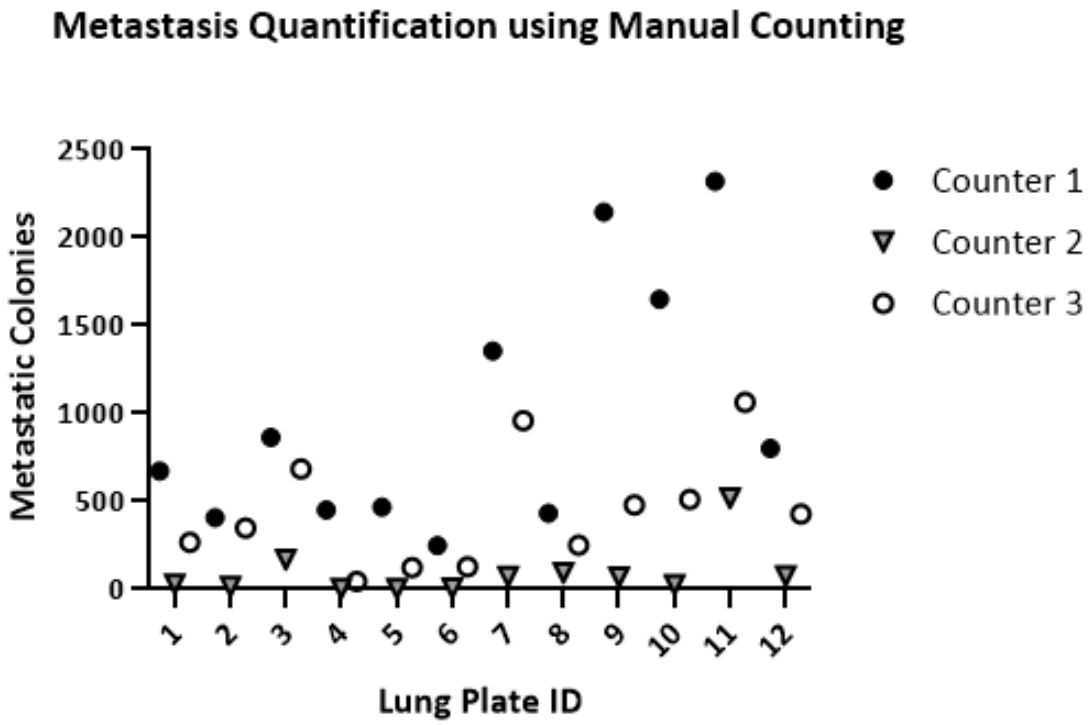
10 (ATCC), A. T. C. C. 4T1 (ATCC CRL-2539) Product Sheet. (2020).

Figure 1 Revisions

Click here to access/download;Figure;Figure 1.2.pdf



A



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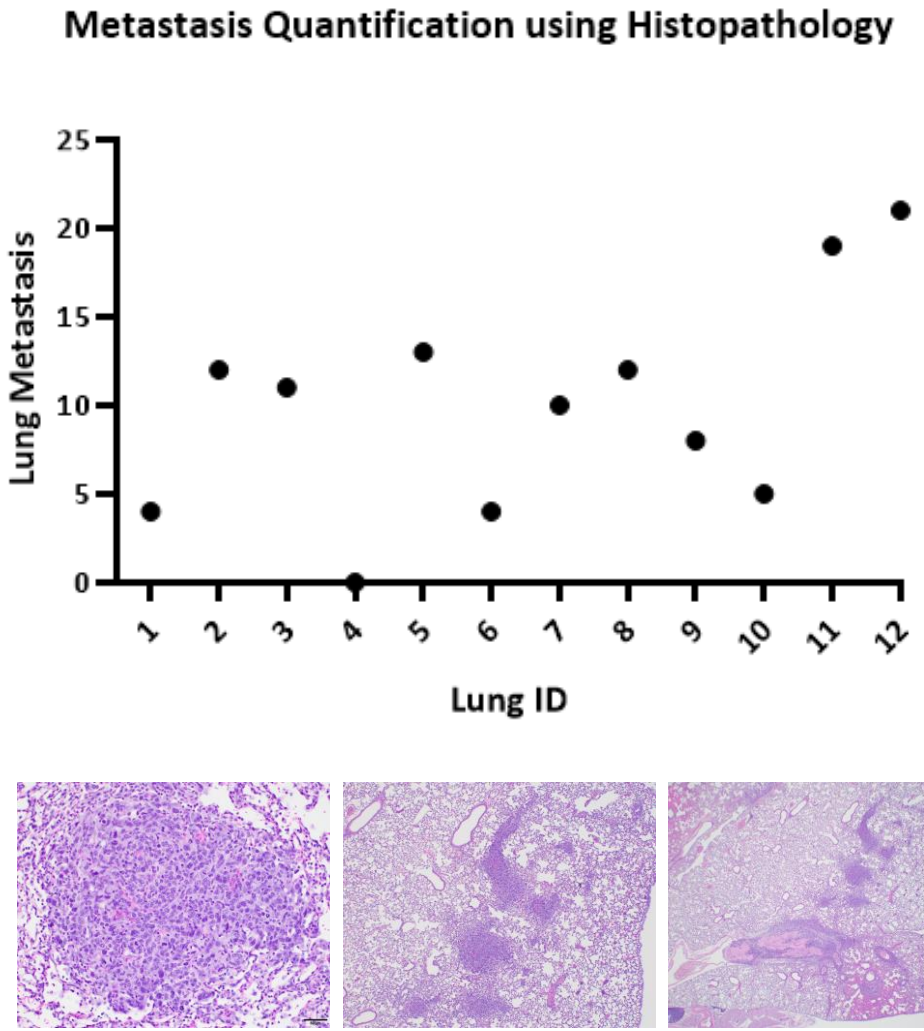
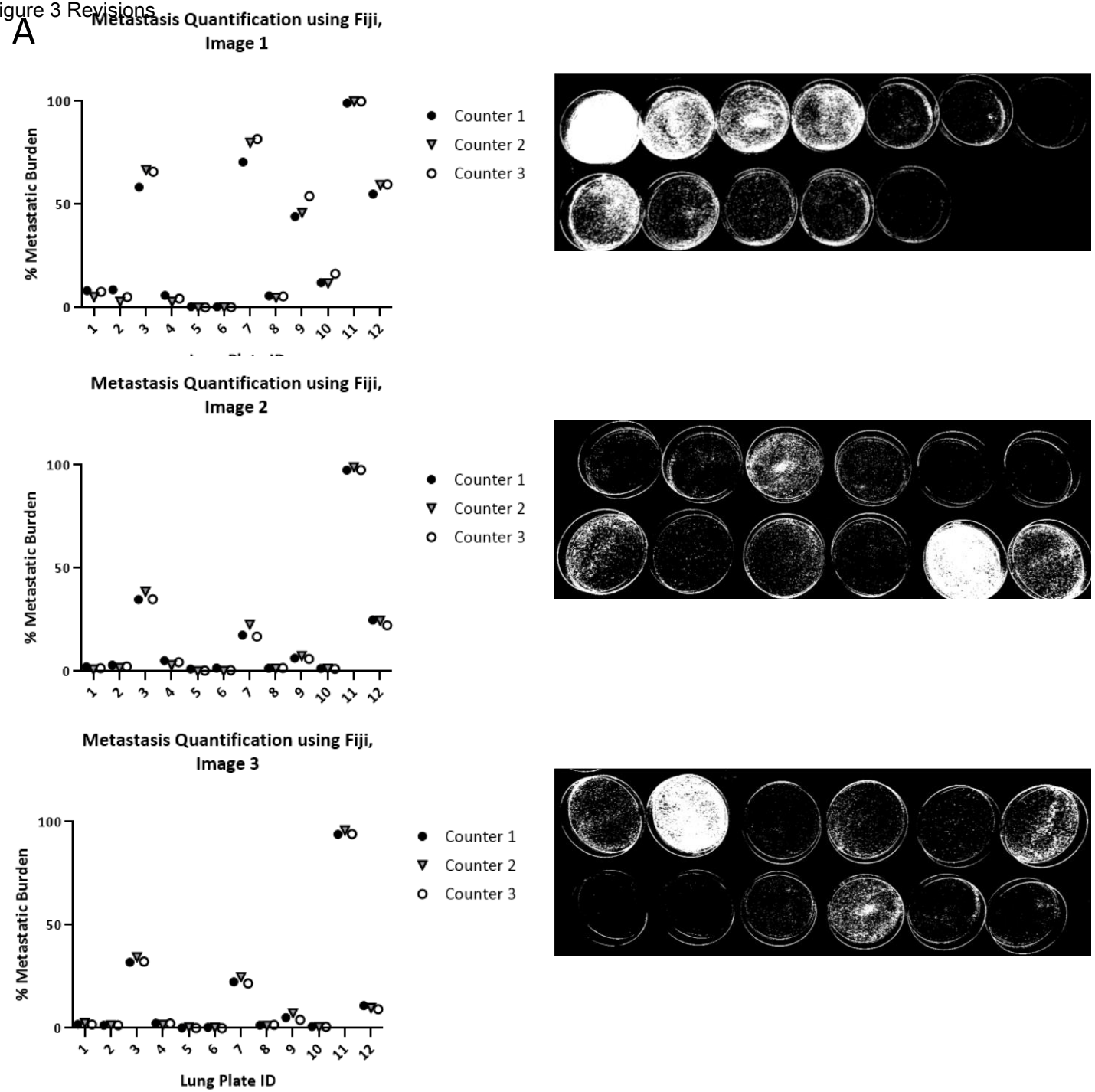


Figure 3 Revisions

Click here to access/download;Figure;Figure 3.2.pdf

A



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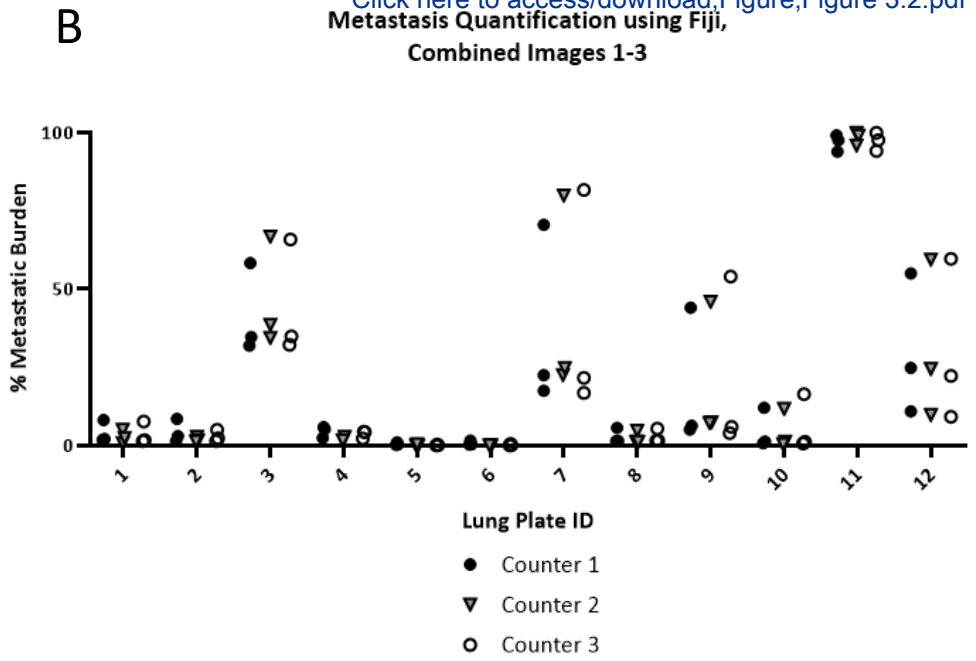


C

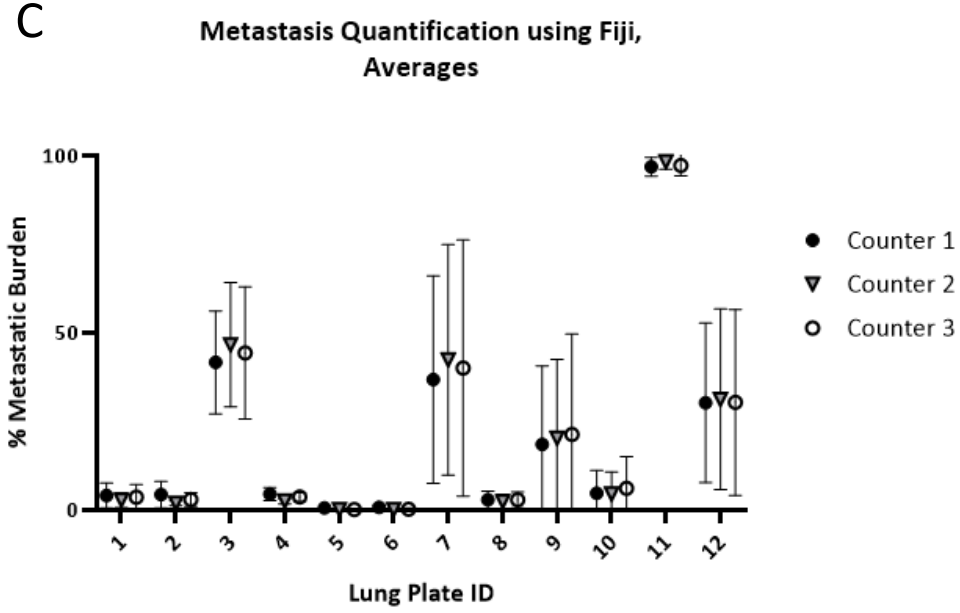


Figure 4 Revisions

A

	Counter 1	Counter 2	Counter 3
Most	11	11	11
	9	3	7
	10	8	3
	7	12	10
	3	7	9
	12	9	12
	1	1	2
	5	10	1
	4	2	8
	8	6	6
	2	4	5
Least	6	5	4

B

	Image 1			Image 2			Image 3		
	Counter 1	Counter 2	Counter 3	Counter 1	Counter 2	Counter 3	Counter 1	Counter 2	Counter 3
Most	11	11	11	11	11	11	11	11	11
	7	7	7	3	3	3	3	3	3
	3	3	3	12	12	12	7	7	7
	12	12	12	7	7	7	12	12	12
	9	9	9	9	9	9	9	9	9
	10	10	10	4	4	4	4	1	4
	2	1	1	2	2	2	1	4	1
	1	8	8	1	8	8	8	2	8
	4	2	2	6	10	1	2	8	2
	8	4	4	8	1	10	10	10	10
	5	6	6	10	6	6	6	5	5
Least	6	5	5	5	5	5	5	6	6

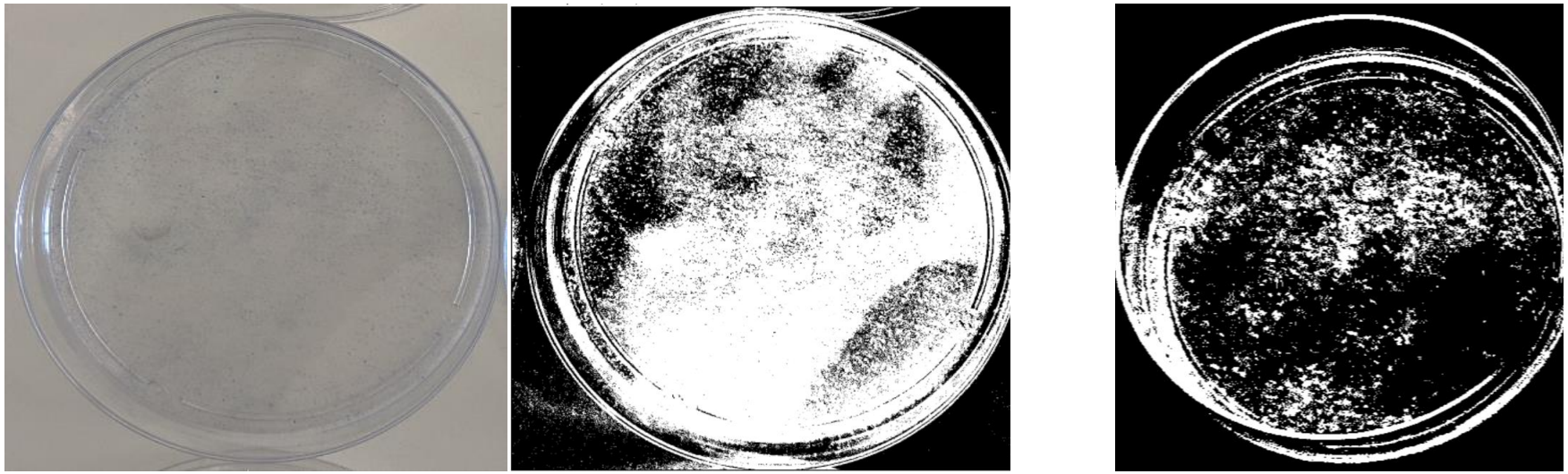
C

	Counter 1	Counter 2	Counter 3
Most	11	11	11
	3	3	3
	7	7	7
	12	12	12
	9	9	9
	10	10	10
	4	1	4
	2	4	1
	1	8	2
	8	2	8
	6	6	6
Least	5	5	5

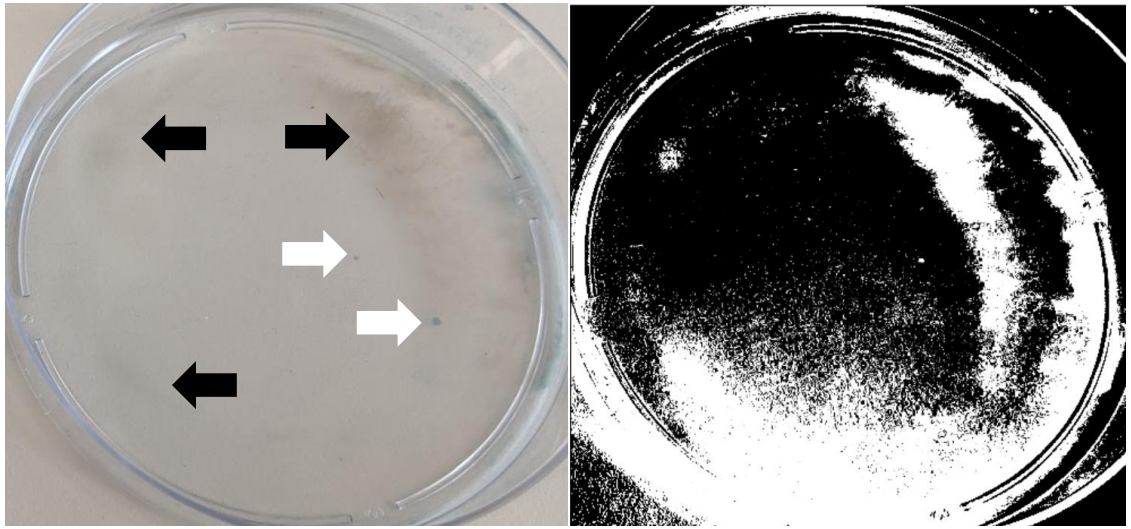
D

	Histopathology
Most	12
	11
	5
	2
	8
	3
	7
	9
	10
	1
	6
Least	4

A



B



Name of Material/ Equipment	Company	Catalog Number
Anesthesia chamber	See comments	See comments
Anesthetic agent	See comments	See comments
BALB/c Female Mice	The Jackson Laboratory	651
Blunt scissors	Roboz	RS-6700
Calculator	Any	Any
Camera	Any	Any
Centrifuge	Any	Any
CO2 euthanasia setup	See comments	See comments
Cold room, refrigerator, cold storage	Any	Any
Computer with Fiji-ImageJ	Any	Any
Counting Chamber	Fisher Scientific	02-671-10
Curved scissors	Roboz	RS-5859
Distilled water	Any	Any
Elastase	MP Biomedicals	100617
Electronic scale	Any	Any
Fetal Bovine Serum (FBS)	R&D Systems	S11150
Forceps	Roboz	RS-8100
Ice	N/A	N/A
Incubator	See comments	See comments
Methanol	Fisher Scientific	A412SK-4
Methylene blue	Sigma-Aldrich	03978-250ML
Penicillin Streptomycin	ATCC	30-2300
Pins or needles	Any	Any
Plastic calipers	VWR	25729-670
RMPI-1640 Medium	ATCC	30-2001
Rocker or rotating wheel	Any	Any
Sharp scissors	Roboz	RS-6702
Sterile disposable filter with PES membrane	ThermoFisher Scientific	568-0010
T-150 Flasks	Fisher Scientific	08-772-48

T-25 Flasks	Fisher Scientific	10-126-10
T-75 Flasks	Fisher Scientific	13-680-65
Tri-cornered plastic beaker	Fisher Scientific	14-955-111F
Trypan blue	VWR	97063-702
Trypsin-EDTA	ATCC	30-2101
Type IV collagenase	Sigma-Aldrich	C5138
1 cm tissue culture plates	Nunclon	153066
1 mL syringe	BD	309659
1.7 mL microcentrifuge tubes	VWR	87003-294
10 cm tissue culture plates	Fisher Scientific	08-772-22
12 well plate	Corning	3512
15 mL centrifuge tube	Fisher Scientific	14-959-70C
1X Dulbecco's Phostphate Buffered Saline (DPBS)	Fisher Scientific	SH30028FS
1X Hank's Balanced Saline Solution (HBSS)	Thermo Scientific	SH3026802
27 g 1/2 in needles	Fisher Scientific	14-826-48
4T1 (ATCC® CRL-2539™)	ATCC	CRL-2539
50 mL centrifuge tube	Fisher Scientific	14-959-49A
6-Thioguanine	Sigma-Aldrich	A4882
70 µM cell strainer	Fisher Scientific	22-363-548
70% ethanol	Sigma Aldrich	E7023

Comments/Description

Use approved materials in your institution's policies

Use approved materials in your institution's policies

Minimum of 8 megapixels

Needs to be capable of 125 x g and 300 x g

Use approved materials in your institution's policies

Needs to be capable of running Fiji-ImageJ

Needs to be capable of 5% CO_2 and 37 °C

For pinning down mice during necropsy

Used to weigh mice

Dilute to 70% with DI water

General Response to the Reviewers and Editors:

The authors would like to express our gratitude for the reviewers and editors for the time they dedicated to reviewing this manuscript. We greatly appreciate the opportunity to improve our manuscript based on the comments we received. Our specific responses are included below, and all changes made to the manuscript are highlighted in the document following the Journal's guidelines. Overall, we included histopathological analysis, clarified other aspects of the protocol, and amended which steps should be included in the video. In general, we believe that the revised manuscript is significantly improved over the original submission.

Editorial Comments:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.
Response:
Thank you for the opportunity to fix any errors. The authors have checked for spelling and grammatical errors.

• Avoid punctuating the title.
Response:
The title has been changed to "Using computer-based image analysis to improve quantification of lung metastasis in the 4T1 breast cancer model" to avoid punctuation.

• Protocol Detail: 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 ensure that all specific details (e.g. button clicks for software actions, numerical values for settings, etc) have been added 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.
Examples:
1) 2.4: How is depth of anesthesia confirmed?
2) 7.2: Mention camera specifications.
Response:
1) This step was amended to include how to monitor depth of anesthesia in lines 166-168.
2) The camera specifications have been added to the Note under 7.2 in lines 300-301.
Other slight wording changes were made to the protocol details to alleviate any vagueness, and these changes are highlighted. Importantly, we have re-evaluated what steps should be shown in the video. The steps highlighted in gray are those we wish to be shown in the video.

• Discussion: JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

Response:
The authors included additional details in the Discussion to elaborate further on these topics. Important to all of these aspects of the Discussion are that we used multiple approaches to the Fiji-ImageJ analysis as discussed in lines 428-465.
1) Modifications and troubleshooting are mentioned in lines 443, 474-477.
2) Limitations are summarized in lines 490-494.
3) Significance with respect to existing methods are mentioned in lines 419-425.
4) Future applications are mentioned in lines 494-500.
5) Critical steps are mentioned in lines 467-472.

• Figure/Table Legends: Define all error bars. Mention statistical tests used.
Response:
Error bars are defined and the statistical test is identified in the Figure Legend of Figure 3. No other figures had statistics run on them as they were not comparing means.

• References: A minimum of 10 references is required.
Response:
Additional relevant references have been added to meet this requirement.

• Table of Materials: Please sort in alphabetical order.
Response:
The table has been sorted in alphabetical order.

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Response:
This is not relevant for our figures.

Comments from Peer-Reviewers:

_____

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
This manuscript gives methodology to quantify distant metastasis using an in vitro culture approach with a standard camera phone and imageJ software. The authors use the 4T1 breast

cancer model which is highly metastatic to the lung. This model frequently forms several metastatic lesions in the lung making accurate quantification by tissue histology difficult and potentially inaccurate. This is a really useful way of quantifying metastasis and the authors correctly point out that this methodology could be used in other organs depending on the tumour model. The equipment, software and protocol are very clear and easy to follow.
Response:
Thank you for this summary. We have included histopathological analysis by a board-certified veterinary pathologist and believe it further confirms the usefulness of our protocol.

Major Concerns:
Using an in vitro approach like this is an alternative to quantification from tissue sections which is very laborious however it would be useful to compare quantification from tissues histology versus in vitro (using both manual and imageJ quantification).
Response:
Thank you for this suggestion. A board-certified veterinary pathologist was added to the paper for analyzing H&E stained lung slides from these mice. We have included the data in our evaluation and discussion as highlighted throughout the manuscript.

The authors describe how the amount of metastasis will vary according to the number of tumour cells originally injected into the mammary fat pad. It would be good to show this quantification methodology from several mice with differing numbers of injected tumour cells.
Response:
This is a great suggestion; however, we feel that it is a bit outside of the scope of this manuscript. Metastasis can be highly variable based on multiple biological factors, including mouse genotype and/or tumor genotype if the 4T1 cells have been altered. Instead of varying the number of cells injected, we chose to use mice of different genotypes, which we felt would better reflect the more common experimental interests of the users of this protocol. The mice used in this manuscript were different genotypes, and were expected to have varying levels of lung metastases. The results demonstrate the expected difference between mice, so therefore we believe that demonstrates the success of this protocol. We mention the different genotypes in line 75-76, but do not elaborate further as we believed it would potentially add confusion.

Minor Concerns:
It would be useful to know if there are upper limits for the maximum density of colonies that imageJ can resolve.
Response:
We agree that this would be useful. Here, we show that Fiji-ImageJ correctly analyzed the most metastatic plate in this manuscript (as obvious to the naked eye) as the most metastatic, so we do not believe we reached an upper limit. Under most experimental conditions, a fully confluent plate would not/should not be included in any analysis as it is likely an outlier. Thus, we believe that we have captured the most likely experimental outcomes.

Using this methodology is it possible to quantify the rates of growth of metastasis?
Response:
It would be possible if mice were sacrificed at different time points during the study. For example, if you wanted to see how quickly the cancer is spreading to the lung, you could sacrifice a set of mice on days 2, 4, 6, 8… post-injection and then analyze using the methodology we present. It could also be used to determine the effect of anti-cancer treatments on lung metastases. These points have been included in lines 495-496.

Reviewer #2:
Manuscript Summary:
The method aims at quantifying the metastatic burden in the lung of a 4T1 breast cancer mouse model. The manuscript reports data from the lung so the title should probably be "(…) in the lung of a 4T1 breast cancer model" as the method cannot provide the complete metastatic burden.
I found the manuscript was clearly written and well organized.
Response:
Thank you for this summary. We have adjusted the title accordingly.

Major Concerns:
For a protocol to be useful, it needs to provide accurate and reproducible results. Unfortunately, the accuracy and the reproducibility of the protocol was not reported.

1) Accuracy: The method would need to be tested against a gold standard method. Note that manual counting of colonies is not appropriate as clearly reported by the authors.
Response:
You are correct in that trusting manual counting as a gold standard is clearly not appropriate. However, manual counting is actually considered the standard of metastasis quantification for this model, based on its description in Current Protocols in Immunology in 2001, and we have clarified this in lines 80-81. We have added histopathology evaluation of H&E stained lung slides by a board-certified veterinary pathologist as another method to compare this protocol to. Please see this added discussion highlighted throughout the manuscript.

2) Reproducibility: The authors could have easily repeated the steps of retaking photographs of existing plates with slightly varying lighting conditions and analyzing the results to demonstrate the method is robust (or not). Counting the same plates from the same images by different users, as reported by the authors, is also a necessary step as it demonstrates how different users can reproduce the image analysis portion of the method.
Response:
We apologize for not making this clearer in the manuscript. We did indeed retake the photographs of the plates 3 different times, as shown by the 3 separate images in Figure 3A. The photographs were taken in 2 separate lab spaces with slightly different lighting. We also

re-arranged the plates in the images or took the image from a different angle for the 3 images used. We have clarified this in lines 348-350 and 357-359.

Minor Concerns:
3) Line 301: My understanding is that the image analysis method relies on an intensity threshold. Surely this is very dependent on lighting conditions.
Response:
You are correct in that lighting would affect the quality of the image, and therefore the analysis. We have specified in lines 295 and 473 that the area should be well-lit. Thresholding is a method to discriminate between the foreground and background. The default threshold command in our protocol tells the software to ignore histogram extremes. As our results for different images, taken in slightly different lighting conditions, were similar, we believe this method is robust.

4) Line 325: The results of Figure 2 differ very widely. Were they trained for the task? It does not make much sense that a counter finds below 100 colonies when another one finds more than 2000.
Response:
Thank you for allowing us to clarify the wide difference between manual counters. We have clarified in the text how the researchers were prepared for counting manually in line 343 and 392-393.

5) Figure 3 B is difficult to read.
Response:
Thank you for bringing this to our attention. We elongated the x axis to help with readability.

6) Line 339: The comparison would be easier if the type of graph was similar between figure 2 and figure 3C.
Response:
The graph has been adjusted to be the same type of graph as Figures 2A, 2B, 3A, and 3B.

7) Line 347: The main weakness of the method is that it is not compared to a gold standard. Manual counting is shown to be highly variable between observers, so this does not constitute a ground truth. Examination of the ranking from least to most metastatic burden is interesting, but it does not constitute a ground truth either.
Response:
Thank you for bringing this concern to our attention. Please see our response to concern #1.

8) Line 368: Here it is mentioned that the researchers were trained to count metastatic colonies the same way. I'm afraid something went very wrong in light of the large difference between their results. It was not mentioned in the text that they had been trained, it was simply mentioned that they were instructed in the same way. Please clarify.
Response:

Thank you for bringing this concern to our attention. Please see our response to concern #4.

9) Line 413 and following. I understand how using a circle selection tool on an image containing all the plates yields more consistent results than cropping images with a variable size and redrawing a circle selection every time. Now, can the authors present the analysis of the same plates using two or three different photographs? This would have informed the reader on the reproducibility of the method.

Response:

Thank you for bringing this concern to our attention. We repeated our procedure for 3 different photographs. Please see our response to concern #2.

10) Figure 3 shows a wide difference in metastatic burden. The protocol appears to be very strict so are these differences due to an expected inter-individual difference or are they due to the proposed protocol?

Response:

This is a great point. The mice used in this manuscript had different genotypes, with differences in lung metastasis between genotypes expected. Using this protocol, there was a significant difference in lung metastasis between genotypes, and therefore solidifies our belief that our method is robust. Inter-individual differences are also expected in this model. We mention the different genotypes in line 75-76, but do not elaborate further as we believed it would add confusion, not clarity.

11) Figure 5. I do not believe the number of significant digits is adequate in Figure 5. Could it be 79.9% and 20.8% in panel A?

Response:

Thank you for pointing this out. We have adjusted the significant figures throughout the manuscript.

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Author(s):	Margaret A Nagai-Singer, Alissa Hendricks-Wenger, Rebecca M Brock, Holly A Morrison, Juselyn D Tupik , Sheryl Coutermarsh-Ott, Irving C Allen

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