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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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Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **Yes**, all done

3. **Interview statements:** Considering the COVID-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group? **Please select one**.

   ☒ Interviewees wear masks until videographer steps away (≥6 ft/2 m) and begins filming, then the interviewee removes the mask for line delivery only. When take is captured, the interviewee puts the mask back on. Statements can be filmed outside if weather permits.

4. **Filming location:** Will the filming need to take place in multiple locations? **No**

Current Protocol Length

Number of Steps: 13
Number of Shots: 32

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Margaret A Nagai-Singer:** This protocol quantifies lung metastasis, which is an aggressive and common cause of breast cancer-related death, with increased precision and efficiency in a preclinical breast cancer model.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

1.2. **Juselyn D Tupik:** Compared to the original technique, this protocol gives researchers quicker, more consistent results. It alleviates human counting error with easy-to-use computer technology.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

OPTIONAL:

1.3. **Alissa Hendricks-Wenger:** This technique can absolutely be extended to preclinical research studying the effect of breast cancer therapies on lung metastasis, by allowing researchers to demonstrate decreased metastatic burden following successful treatment.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

Ethics Title Card

1.4. Procedures involving animal subjects have been approved by the Institutional Animal Care and Use Committee (IACUC) at Virginia Tech.

Protocol

2. Processing Tissues

2.1. Begin by labeling a 15-milliliter conical tube for the tissue sample **[1-TXT]**, then add 2.5 milliliters of type IV *('4')* collagenase mixture and 30 units of elastase to the tube **[2]**.

2.1.1. WIDE: Establishing shot of talent labeling a tube. **TEXT: 1 tube per mouse**

2.1.2. Talent adding the collagenase and elastase to a tube.

2.2. Move the lung to a clean 1x HBSS well and swirl it with forceps to remove any remaining blood **[1]**. Then, transfer it to an empty 3.5-centimeter tissue culture plate **[2]**.

2.2.1. Talent transferring the lung with clean HBSS and swirling. NOTE: This and next shot together

2.2.2. Talent transferring the lung to the culture plate.

2.3. Mince the lung with scissors **[1]**, then rinse the plate with 2.5 milliliters of HBSS **[2]** and transfer the HBSS with the lung pieces into a prepared 15-milliliter conical tube with the collagenase and elastase cocktail **[3]**. *Videographer: This step is important!*

2.3.1. Talent mincing the lungs.

2.3.2. Talent rinsing the plate with HBSS. NOTE: This and next shot together, with a take to show clean plate

2.3.3. Talent transferring the tissue to the conical tube.

2.4. Incubate the tube for 75 minutes at 4 degrees Celsius on a rocker or rotating wheel **[1]**. Meanwhile, label one 50-milliliter centrifuge tube and one 10-centimeter tissue culture plate for each mouse. If doing a dilution, label enough tissue culture plates for the dilutions **[2-TXT]**.

2.4.1. Tube incubating on the rocker.

2.4.2. Talent labeling the lid of a plate. **TEXT: Label the lids**

2.5. Bring the volume of the tube up to 10 milliliters with 1x HBSS **[1]**, then pour the contents over a 70-micrometer cell strainer into the 50-milliliter conical tube **[2]**. Use the plunger of a 1-milliliter syringe to gently grind the sample through the strainer **[3]**.

2.5.1. Talent adding HBSS to a tube to bring the volume to 10mL.

2.5.2. Talent pouring the contents into the strainer. NOTE: This and next shot together

2.5.3. Talent pushing the sample into the 50mL tube.

2.6. Centrifuge the tube for 5 minutes at 350 x *g* **[1]** and discard the supernatant **[2]**, then wash the pellet twice with 10 milliliters of 1x HBSS **[3]**.

2.6.1. Talent putting the tube in the centrifuge and closing the lid.

2.6.2. Talent discarding the supernatant.

2.6.3. Talent adding HBSS to the pellet.

2.7. Resuspend the pellet in 10 milliliters of 60 micromolar 6TG complete culture media **[1-TXT]** and plate samples in the 10-centimeter cell culture plates, using a dilution scheme if desired **[2]**. Incubate the plates at 37 degrees Celsius and 5% carbon dioxide for 5 days **[3]**.

2.7.1. Talent resuspending the pellet in media, with the media container in the shot. **TEXT: CAUTION! 6TG is toxic** NOTE: This and next shot together

2.7.2. Talent plating the cells in the culture plate.

2.7.3. Talent putting the plates in the incubator and closing the door.

3. Staining Plates

3.1. Pour culture media off of the plates into the appropriate waste container **[1]**. To fix the cells, add 5 milliliters of undiluted methanol to each plate and incubate for 5 minutes at room temperature **[2-TXT]**, making sure to swirl the methanol so that it covers the entire plate **[3]**. *Videographer: This step is important!*

3.1.1. Talent pouring off media.

3.1.2. Talent adding methanol to a plate, with the methanol container in the shot. **TEXT: CAUTION! Methanol is hazardous. Work under a fume hood**

3.1.3. Talent swirling a plate.

3.2. Pour the methanol off the plates into an appropriate waste container **[1]**, then rinse each plate with 5 milliliters of distilled water **[2]**. Add 5 milliliters of 0.003% methylene blue per plate and incubate it for 5 minutes at room temperature, making sure to swirl the methylene blue solution so that it covers the entire plate **[3]**. *Videographer: This step is important!*

3.2.1. Talent pouring the methanol off a plate.

3.2.2. Talent rinsing the plate with water.

3.2.3. Talent adding methylene blue to a plate and swirling it.

3.3. Pour the methylene blue into the appropriate waste container **[1]** and rinse each plate again with 5 milliliters of distilled water **[2]**. Turn the plate upside down and blot it

against a paper towel to remove excess liquid **[3]**. Place the plate on its lid and let it air dry overnight **[4]**. *Videographer: This step is important!*

3.3.1. Talent pouring off methylene blue.

3.3.2. Talent rinsing the plate with water.

3.3.3. Talent turning the plate upside down on a paper towel.

3.3.4. Talent placing the plate on its lid and leaving it to dry.

3.4. Metastatic colonies will be blue. Once the plates have dried, they can be stored at room temperature indefinitely **[1]**. *Videographer: This step is important!*

3.4.1. Plate with blue colonies.

4. Image analysis

4.1. Remove the labeled lids from plates, taking care to ensure clear identification of the samples **[1]**. Line up all stained lung plates on a clean, light surface **[2]**.

4.1.1. Talent removing lids from plates.

4.1.2. Talent lining the plates up on an appropriate surface.

4.2. Take a picture of the collection of plates in a well-lit area, making sure to minimize reflections. Reflections in the plates will influence image analysis and should be avoided **[1]**. Pay close attention to the photographs taken and take several pictures from several angles **[2]**. *Videographer: This step is important!*

4.2.1. Talent photographing the plates.

4.2.2. Talent looking through the photographs.

4.3. After photographing the plates, crop the image to exclude the lids or anything in the background **[1]**.

4.3.1. SCREEN: 61805_screenshot_1.mp4.

4.4. Open the image in Fiji-ImageJ and change it to black and white by clicking Image, Adjust, and Color Threshold. Then, select Default for Thresholding method, black and white for Threshold color, and Lab for Color space. Make sure that the Dark background box is not selected **[1]**.

4.4.1. SCREEN: 61805_screenshot_2.mp4. 0:00 – 0:13.

4.5. The image should now be black and white. Black represents the light background, and white represents the blue metastatic colonies **[1]**.

4.5.1. SCREEN: 61805_screenshot_2.mp4. 0:13 – 0:15.

4.6. Use the Circle tool to 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 the analyzed area on the plates while minimizing the background noise that appears on the edge of the plates **[1]**. The size appears in the toolbar as it is drawn **[2]**.

4.6.1. SCREEN: 61805_screenshot_3.mp4. 0:00 – 0:25.

4.6.2. SCREEN: 61805_screenshot_3.mp4. 0:25 – end. *Video Editor: Emphasize the circle size in the bottom left corner.*

4.7. Analyze the selected circle to determine the percentage of area that is white. Click on Analyze and Analyze Particles **[1]**, then select 0 to infinity for Size, 0 to 1 for Circularity, and Nothing for Show. Check the Summarize box and hit OK **[2]**.

4.7.1. SCREEN: 61805_screenshot_4.mp4. 0:00 - 0:03.

4.7.2. SCREEN: 61805_screenshot_4.mp4. 0:03 – end. *Video Editor: Emphasize the Size, Circularity, and Show selections and the Summarize box.*

4.8. Move the circle to the next plate in the picture by grabbing its center and repeat the analysis **[1]**.

4.8.1. SCREEN: 61805_screenshot_5.mp4. *Video Editor: A few examples of this process shown one after the other, we only need to show one.*

4.9. Copy and paste the result into a spreadsheet. The percent Area, which is the percentage of the selected area that is white, represents the metastatic burden **[1]**.

4.9.1. SCREEN: 61805_screenshot_6.mp4.

4.10. Once all plates and images have been analyzed, average the percent Area results between different images for each plate to mitigate any inconsistencies between pictures **[1]**.

4.10.1. SCREEN: 61805_screenshot_7.mp4.

Results

5. Results: Fiji-ImageJ Analysis is Accurate and Precise in Determining Metastatic Burden

5.1. Fiji-ImageJ analysis was compared to manual counting **[1]** and histopathological analysis **[2]**. When 3 separate researchers manually counted metastatic colonies, the results were inconsistent between counters **[3]**.

5.1.1. LAB MEDIA: Figure 2 A.

5.1.2. LAB MEDIA: Figure 2 B.

5.1.3. LAB MEDIA: Figure 2 A. *Video Editor: Emphasize the circles and triangles, the goal is to show how spread out the results are.*

5.2. Fiji-ImageJ results were consistent between counters for each of the 3 images **[1]**.

5.2.1. LAB MEDIA: Figure 3 A.

5.3. The results from the 3 images and the 3 counters were combined for each lung plate **[1]**.

5.3.1. LAB MEDIA: Figure 3 B.

5.4. The results were averaged for each plate to account for the variations between the images, which provided consistent results between counters **[1]**.

5.4.1. LAB MEDIA: Figure 3 C.

5.5. When ranking the plates from most to least metastatic, manual counting agreed on the most confluent plate **[1]**, but all following ranks were inconsistent **[2]**. The ranks from the averaged Fiji-ImageJ results were much more consistent between counters **[3]**.

5.5.1. LAB MEDIA: Figure 4 A. *Video Editor: Emphasize the top row.*

5.5.2. LAB MEDIA: Figure 4 A. *Video Editor: Emphasize all rows except the top.*

5.5.3. LAB MEDIA: Figure 4 C.

5.6. 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 **[1]** along with an image of the same plate without a reflection **[2]**.

5.6.1. LAB MEDIA: Figure 5 A. *Video Editor: Emphasize the left images.*

5.6.2. LAB MEDIA: Figure 5 A. *Video Editor: Emphasize the right image.*

5.7. Dark blemishes from a dirty background surface or blood sample residue on the plates can also negatively impact Fiji-ImageJ analysis. This blood plate only has 2 metastatic colonies **[2]**, but the dark residue caused Fiji-ImageJ to consider it as 31.6% metastatic **[3]**.

5.7.1. LAB MEDIA: Figure 5 B.

5.7.2. LAB MEDIA: Figure 5 B. *Video Editor: Emphasize where the white arrows are pointing.*

5.7.3. LAB MEDIA: Figure 5 B. *Video Editor: Emphasize where the black arrows are pointing.*

Conclusion

6. Conclusion Interview Statements

6.1. **Holly A Morrison:** When attempting this protocol, remember to carefully examine pictures for reflections, use the same-sized circle for each plate in the image, and average the results for each plate between at least 3 separate images.

6.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 4.2.1, 4.2.2, 4.6.1, 4.10.1*