Journal of Visualized Experiments

# How to generate murine orthotopic metastatic breast cancer model and perform murine radical mastectomy

--Manuscript Draft--

| | |
|---|---|
| **Article Type:** | Invited Methods Article - JoVE Produced Video |
| **Manuscript Number:** | JoVE57849R2 |
| **Full Title:** | How to generate murine orthotopic metastatic breast cancer model and perform murine radical mastectomy |
| **Keywords:** | Mouse, Breast, Cancer, Model, Orthotopic, Syngeneic, Mastectomy, Metastasis, Bioluminescence, IVIS |
| **Corresponding Author:** | Eriko Katsuta<br>Roswell Park Cancer Institute<br>Buffalo, New York UNITED STATES |
| **Corresponding Author's Institution:** | Roswell Park Cancer Institute |
| **Corresponding Author E-Mail:** | Eriko.Katsuta@roswellpark.org |
| **Order of Authors:** | Eriko Katsuta |
| | Omar M. Rashid |
| | Kazuaki Takabe |
| **Additional Information:** | |
| **Question** | **Response** |
| Please indicate whether this article will be Standard Access or Open Access. | Standard Access (US$2,400) |
| Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations. | Elm and Carlton Streets, Buffalo, NY, 14263 |

Cover Letter

ROSWELL PARK CANCER INSTITUTE
Elm & Carlton Streets | Buffalo, NY 14263
716-845-2300 | www.roswellpark.org
E-mail: askrpci@roswellpark.org

UNDERSTAND PREVENT & CURE CANCER

**Kazuaki Takabe, MD, PhD, FACS**
Professor and Alfiero Foundation Chair of Breast Oncology, Clinical Chief of Breast Surgery
Roswell Park Cancer Institute
Ph: 716-845-5540, Fax: 716-845-5705, Kazuaki.Takabe@RoswellPark.org

Professor of Surgery, University at Buffalo the State University of New York
Affiliated Professor, Tokyo Medical University and Yokohama City University
Liaison Professor, Niigata University Graduate School of Medicine and Dentistry

Aug 10, 2018

Dr. Alisha DSouza
Senior Review Editor
Journal of Visualized Experiments (JoVE)

Dear Dr.DSouza,

Thank you for the comprehensive and helpful review of our manuscript entitled "**How to generate murine orthotopic metastatic breast cancer model and perform murine radical mastectomy.**" Please find the point-by-point response to the Editorial comments below. We have revised the manuscript accordingly.

We hope that these revisions are satisfactory and that the manuscript is now acceptable for publication in *Journal of Visualized Experiments (JoVE)*.

Sincerely,

Kazuaki Takabe, M.D., Ph.D., FACS
Professor, Alfiero Foundation Chair and Clinical Chief of Breast Surgery
Roswell Park Cancer Institute

**TITLE:**

# Generating a Murine Orthotopic Metastatic Breast Cancer Model and Performing Murine Radical Mastectomy

**AUTHORS:**

Eriko Katsuta[1], Omar M. Rashid[2,3,4,5], Kazuaki Takabe[1,6,7,8,9,10]

[1]Breast Surgery, Department of Surgical Oncology, Roswell Park Comprehensive Cancer Center, Buffalo, NY, United States of America
[2]Holy Cross Hospital Michael and Dianne Bienes Comprehensive Cancer Center, Fort Lauderdale, FL, United States of America
[3]Department of Surgery, Massachusetts General Hospital, Boston, MA, United States of America
[4]Department of Surgery, University of Miami Miller School of Medicine, Miami, FL, United States of America
[5]Department of Surgery, Nova Southeastern University School of Medicine, Fort Lauderdale, FL, United States of America
[6]Department of Surgery, University at Buffalo Jacobs School of Medicine and Biomedical Sciences, The State University of New York, Buffalo, NY, United States of America
[7]Department of Breast Surgery and Oncology, Tokyo Medical University, Tokyo, Japan
[8]Department of Surgery, Yokohama City University, Yokohama, Japan
[9]Department of Surgery, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan
[10]Department of Surgery, Fukushima Medical University, Fukushima, Japan

**Corresponding Author:**

Kazuaki Takabe (kazuaki.takabe@roswellpark.org)

**E-mail Addresses of the Co-authors:**

Eriko Katsuta (eriko.katsuta@roswellpark.org)
Omar M. Rashid (omar.rashid@holy-cross.com)

**KEYWORDS:**

Mouse, breast, cancer, model, orthotopic, syngeneic, mastectomy, metastasis, bioluminescence, IVIS

**SHORT ABSTRACT:**

We introduce a murine orthotopic breast cancer model and radical mastectomy model with bioluminescence technology to quantify the tumor burden to mimic human breast cancer progression.

**LONG ABSTRACT:**

*In vivo* mouse models to assess breast cancer progression are essential for cancer research, including preclinical drug developments. However, the majority of the practical and technical details are commonly omitted in published manuscripts which, therefore, makes it challenging

to reproduce the models, particularly when it involves surgical techniques. Bioluminescence technology allows for the evaluation of small amounts of cancer cells even when a tumor is not palpable. Utilizing luciferase-expressing cancer cells, we establish a breast cancer orthotopic inoculation technique with a high tumorigenesis rate. Lung metastasis is assessed utilizing an *ex vivo* technique. We, then, establish a mastectomy model with a low local recurrence rate to assess the metastatic tumor burden. Herein, we describe, in detail, the surgical techniques of orthotopic implantation and mastectomy for breast cancer with a high tumorigenesis rate and low local recurrence rates, respectively, to improve breast cancer model efficiency.

**INTRODUCTION:**

Animal models play a key role in cancer research. When a hypothesis is proven *in vitro*, it should be tested *in vivo* to evaluate its clinical relevance. Cancer progression and metastasis are often better captured by animal models as compared to *in vitro* models, and it is essential to test a new drug in an animal model as a preclinical study for drug development[1,2]. However, the technical details of animal experiments are often not well described in published articles, making it challenging to reproduce the model successfully. Indeed, the authors who established these orthotopic inoculation and mastectomy models went through long and rigorous processes of trial and error. The success rate of tumorigenesis after cancer cell inoculation is one of the key factors to determine the success and efficiency of an animal study[3]. The cell line and the number of cells to inoculate, the inoculation site, and the strain of the mice are all important factors. It is well known that there are huge variations in the results of animal experiments due to individual differences, compared to *in vitro* techniques. Therefore, using a well-established model with a standard technique is important to obtain stable results, to improve the efficiency of animal experiments, and to avoid misleading results.

This paper provides well-established techniques[4] to generate breast cancer orthotopic and mastectomy mouse models. The aims of these methods are 1) to mimic human breast cancer progression and treatment courses, and 2) to conduct *in vivo* experiments with greater efficiency and higher success rates compared to other breast cancer inoculation or mastectomy techniques. In orthotopic cancer cell inoculation, to mimic human breast cancer progression, we choose the #2 mammary fat pad as an inoculation site, which is located in the chest. In most of the studies, breast cancer cells are inoculated subcutaneously[5]. This technique does not require surgery and, thus, it is simple and straightforward. However, the subcutaneous microenvironment is quite different from the mammary gland microenvironment, which results in different cancer progression and even molecular profiles[6,7]. Some studies use the #4 mammary gland, which is located in the abdomen, as an inoculation site[6]. However, since #4 mammary glands are located in the abdomen, the most common metastatic pattern is peritoneal carcinomatosis[7], which occurs with less than 10% of metastatic breast cancer[8]. Breast cancer generated by the technique presented here, in the #2 mammary gland, metastasizes to the lung, which is one of the most common breast cancer metastatic sites[9].

With this technique, the goal is also to achieve a higher tumorigenesis rate with minimal tumor size variability compared to other breast cancer inoculation techniques. To do so, cancer cells suspended in a gelatinous protein mixture are inoculated under direct vision through a median

anterior chest wall incision. This technique produces a high tumorigenesis rate with less variability in tumor size and shape compared to subcutaneous or non-surgical injection, as previously reported[3,7].

We also introduce a mouse radical mastectomy technique in which the orthotopic breast tumor is resected with the surrounding tissues and axillary lymph nodes. In the clinical setting, the standard of care for breast cancer patients without distant metastasis disease is mastectomy[10,11]. Before a mastectomy, axillary lymph node metastasis is surveyed by imaging and sentinel lymph node biopsy. If there is no evidence of axillary lymph node metastasis, the patient is then treated with a total or partial mastectomy, in which the axillary lymph node resection is omitted. Total mastectomy is a technique to resect breast cancer with the whole breast tissue en bloc, whereas partial mastectomy is to resect breast cancer with a margin of surrounding normal breast tissue only, thus conserving the remaining normal breast tissue in the patient. However, patients who preserve remaining normal breast tissue after a partial mastectomy require postoperative radiotherapy to avoid local recurrence[10]. Patients who have axillary lymph node metastasis undertake radical mastectomy which removes the breast cancer with all normal breast tissue and axillary lymph nodes and invaded tissues en bloc[10,11]. In the mouse model, surveillance for axillary lymph node metastasis and/or post-operative radiation is not reasonable or feasible. Thus, we utilize the radical mastectomy technique to avoid local or axillary lymph node metastasis.

Cancer cell inoculation *via* the tail vein is the most common lung metastasis mouse model[12], the so-called "experimental metastasis". This model is easy to generate and does not require surgery; however, it does not mimic human breast cancer progression which may result in different metastatic disease behavior. In order to mimic the human breast cancer treatment course where metastasis often occurs after mastectomy, the primary tumor is removed after orthotopic cancer cell inoculation. This technique produces less local recurrence compared to simple tumor resection, as previously reported[13], and is useful for novel therapeutics, preclinical studies, and for metastatic breast cancer research studies. The techniques described here are applicable for most breast cancer orthotopic model experiments. However, it is important to consider that the gelatinous protein mixture can affect the microenvironment and surgery can affect the stress/immune response[14]. Therefore, investigators studying the microenvironment and/or the stress/immune response should be aware of potential confounding factors.

**PROTOCOL:**

Approval from the Roswell Park Comprehensive Cancer Center Institutional Animal Care and Use Committee was obtained for all experiments.

Note: Nine- to twelve-weeks-old female BALB/c mice are obtained. 4T1-luc2 cells, a mouse mammary adenocarcinoma cell line derived from BALB/c mice that has been engineered to express luciferase, are used. These cells are cultured in Roswell Park Memorial Institute (RPMI) 1640 medium with 10% fetal bovine serum (FBS).

**1. Preparation of Instruments**

1.1) Thaw a frozen gelatinous protein mixture (*e.g.*, Matrigel) on ice in a tissue culture hood.

1.2) Clean and autoclave two sets of surgical instruments (microdissection scissors, Adson forceps, and a needle holder) prior to surgery. Prepare sterilized 5-0 silk sutures and dry sterilant (when serial surgeries are planned).

1.3) Clip the middle chest hair of the mice using a clipper and mark the mice for identification by punching the ear prior to the time of surgery.

1.4) Prepare the procedure table, which can be immediately used for the operation.

1.4.1) Spread an absorbent pad and fix the corners with tape, fix anesthesia nose cones with tape, put sterilant and disinfectant (chlorhexidine, iodine, and 75% ethanol) beside the operating space, and place the mice in operation order.

**2. Preparation of Cells (for 10 Mice)**

Note: The cells should be inoculated within 1 h after being detached from the dish to avoid decreased cell viability. Specifically, the cell suspension should be mixed into the gelatinous protein mixture within 15 min after detaching the cells from the dish to maintain their viability.

2.1) Culture 4T1-luc2 cells, a mouse mammary adenocarcinoma cell line expressing luciferase, in RPMI 1640 media with 10% FBS in a humidified incubator at 37 °C in 5% $CO_2$.

2.2) Wash the adherent 4T1-luc2 cells in a 10-cm dish with phosphate-buffered saline (PBS) using a 10-mL serological pipette. Add 1 mL of 0.25% trypsin using a P1000 pipette and, then, incubate the sample at 37 °C for 5 min. Then, add 4 mL of growth media (RPMI-1640 with 10% FBS) using a 5-mL serological pipette and transfer the cell suspension to a 15-mL conical tube in a tissue culture hood. Centrifuge the cell suspension at 180 x *g* for 5 min.

2.3) Aspirate the supernatant and resuspend the cells in 2 mL of PBS; then, count the cells using the hemocytometer.

2.4) Suspend 2 x $10^6$ 4T1-luc2 cells in 40 µL of cold PBS (pH 7.4, 4 °C) in the tissue culture hood.

2.5) Mix the 40-µL cell suspension with 360 µL of the gelatinous protein mixture in a 1.5-mL microcentrifuge tube on ice in the tissue culture hood.

Note: The final concentration is 1 x $10^5$/20 µL (1:9 PBS:gelatinous protein mixture). For the orthotopic model (no mastectomy), 1 x $10^4$/20 µL of the final concentration was used, to avoid reaching euthanasia criteria (tumor size > 2 cm) within two weeks.

**3. Cancer Cell Inoculation**

3.1) Put the mice in the anesthesia induction chamber with 2.5% isoflurane and 0.2 L/min oxygen flow until the mice breathe calmly (2 - 3 min).

3.2) Grasp the mouse and inject 0.05 mg/kg buprenorphine into its shoulder subcutaneously.

3.3) Confirm adequate anesthesia by the lack of reaction to a toe pinch. Insert the mouse's nose into the hole of the mouse mask, which allows inhalational anesthesia with 2.5% isoflurane and 2 L/min oxygen flow attached to a charcoal canister unit.

3.4) Restrain the mouse's limbs using lab tape and sterilize its skin using chlorhexidine, iodine, and 75% ethanol, using cotton swabs.

3.5) Make a 5-mm skin incision in the middle of the anterior chest wall utilizing sterile microdissection scissors, lift the right-side skin next to the incision and detach the skin from the chest wall using the scissors, and then, invert the skin to expose the right #2 mammary fat pad.

3.6) Carefully inject 20 µL of cancer cell suspension using a 1-mL insulin syringe with a 28.5-G needle into the fat pad under direct vision through the wound.

Note: The needle goes through the wound, not the skin. Keep holding the needle in the fat pad for 5 s prior to pulling it out, which allows time for the gelatinous protein mixture to solidify.

3.7) Close the skin incisions by stitching, using sterile 5-0 non-absorbable sutures.

3.8) After surgery, return the animals to a clean cage and monitor them until they have recovered and are moving freely (after ~1 - 2 min).

3.9) Remove the sutures under anesthesia (see step 3.1) 7 d after the surgery.

**4. Mastectomy**

Note: The timing of the mastectomy is very important. If it is done too early, lung metastasis does not occur. If it is done too late, the primary tumor has invaded major blood vessels, which make a complete oncologic resection challenging. Thus, multiple time points were tested for mastectomy to determine which time point produced the appropriate balance in waiting for metastasis before resection became too challenging. After doing so in over 50 mouse experiments, it was demonstrated that mastectomy at 8 d after cancer cell inoculation (or when the tumor size reaches 5 mm) was the ideal time point to achieve that balance[13].

4.1) Anesthetize a mouse with 2.5% inhaled isoflurane and inject buprenorphine (see steps 3.1 and 3.2).

4.2) Restrain the mouse and sterilize its skin (see step 3.4).

4.3) Make a 5-mm skin incision 2 mm to the left from the surgical scar that was made at the initial cancer cell inoculation, using the microdissection scissors. Extend the incision toward the root of the forelimb to remove the tumor, the skin including the surgical scar, and the lesion in contact with the tumor, as well as the axillary lymph node basin in which most of the time no visible lymph node exists at the time of the mastectomy[13]. Make sure not to damage the axillary vein.

4.4) Close the skin defects by stitching, using sterile 5-0 non-absorbable sutures in the shape of a "Y".

4.5) The same as in step 3.8, return the mouse to a clean cage and monitor until they have recovered.

4.6) Remove the sutures under anesthesia (see step 3.1) 7 d after surgery.

**5. Bioluminescent Quantification of the Primary Tumor (Orthotopic Inoculation Without Mastectomy) or Lung Metastasis (Mastectomy Model)**

Note: For primary tumor burden quantification, the bioluminescence is measured 2x a week from the day after the orthotopic inoculation. For lung metastasis quantification, the bioluminescence is measured 2x a week from the day after the mastectomy.

5.1) Dissolve D-luciferin in Dulbecco's phosphate-buffered saline (DPBS) to a final concentration of 15 mg/mL in a tissue culture hood. Aliquot it into light-shielded 1.5-mL microcentrifuge tubes. Store the diluted solution at -80 °C.

Note: For a 20-g mouse, 200 µL of diluted D-luciferin is required.

5.2) Open the imaging software and click **Initialize**.

Note: It takes about 15 min to cool the charge-coupled device (CCD). When the CCD reaches the set temperature, the color of the **Temperature** bar changes from red to green.

5.3) Anesthetize the mice with 2.5% isoflurane in a dedicated induction chamber prior to imaging (see step 3.1).

5.4) Weigh the mice.

5.5) Inject 150 mg/kg D-luciferin intraperitoneally at the point of the mid-abdomen, using a 28.5-G needle.

5.6) Fit each mouse with a nose cone inside the imaging system in the supine position (place a maximum of five mice at the same time). Maintain anesthesia at 1% - 3% isoflurane (in 100% oxygen) through the nose cones during imaging.

5.7) Capture an image every 5 min to detect the peak bioluminescence for 50 min (or up to the confirmed peak bioluminescence).

5.7.1) Select **Luminescence** as **Auto**, **Binning** as **Medium**, and **Field of View** as **D**.

5.7.2) Click **Acquire** to capture the image.

5.8) Return the mice to their cage(s) and monitor them until they have recovered (see step 3.8).

## 6. Lung Metastasis Tumor Burden Quantification by *Ex Vivo* Imaging

Note: Lung metastasis quantification is applicable for orthotopic inoculation both with and without mastectomy models. In the mastectomy model, *ex vivo* imaging or survival observation is chosen, depending on the purpose. In the orthotopic inoculation (without mastectomy) model, most cases produce primary tumor size euthanasia criteria (> 2 cm) approximately 21 d after inoculation.

6.1) Quantify lung metastatic lesions 21 d after the cancer cell inoculation by *ex vivo* imaging.

6.2) Anesthetize the mice with 2.5% isoflurane in a dedicated induction chamber (see step 3.1).

6.3) Weigh the mice.

6.4) Inject 150 mg/kg D-luciferin intraperitoneally (see step 5.6).

6.5) Euthanize the mice by cervical dislocation, 15 min after the injections.

6.6) Open the abdomen by cutting the skin and peritoneum at the mid-abdomen, using curved Mayo scissors. Extend the incision to both the right and the left. Pull out the liver with forceps until the diaphragm is visualized; then, cut the diaphragm.

6.7) Using the curved Mayo scissors, cut the bilateral ribs from caudad (12th ribs) to cephalad (1st ribs) to expose the lungs by flipping the anterior thorax wall.

6.8) Identify the thoracic esophagus, which looks like a cord connecting the lungs to the spine, by lifting the lungs using forceps and, then, cut the esophagus using the microdissection scissors.

6.9) Lift the bilateral lungs and heart using forceps (applying traction pulling down, in the direction of cephalad to caudad) and, then, cut the trachea and major vessels to the lung apex at the cephalad, using microdissection scissors.

Note: This allows for the isolation of the lung and heart from the body.

6.10) Remove the heart from the lung using microdissection scissors.

6.11) Put the lungs in a 10-cm Petri dish.

6.12) Capture the bioluminescence image (see steps 5.7.1 and 5.7.2) 5 min after euthanasia (20 min after the luciferin injection).

**REPRESENTATIVE RESULTS:**

The purpose of the orthotopic model is to mimic human cancer progression (*i.e.*, the growth of the primary tumor followed by lymph node metastasis and then distant lung metastasis)[15]. After cancer cell inoculation, the bioluminescence is quantified regularly (two to three times/week) (**Figure 1A**). The bioluminescence in the lungs is deeper and smaller than the primary lesion. The bioluminescence mainly reflects the primary tumor burden in live mice[3] (**Figures 1B** and **1C**). The tumor size was also measured by caliper measurement. The tumor volume was estimated using the following equation: volume = (length) x $(width)^2/2$ (**Figure 1D**). Quantification of the tumor burden by bioluminescence and caliper measurement showed similar trends in the representative results (**Figures 1B** and **1D**); however, we sometimes encountered discrepancies. We assume that quantifying the primary tumor burden utilizing bioluminescence is more accurate compared to measuring the tumor size by caliper when the tumor size is less than 1.5 cm. Because it reflects viable cancer cells, even a small number of cells can be detected by bioluminescence, and only cancer cells can be detected inside of the tumor, not including infiltrating immune cells or surrounding stromal cells[3]. This model is useful in evaluating the efficacy of tumor regression mediated by anti-cancer drug and immune responses[3,7,16]. The tumor burden of the lung metastases can also be quantified by *ex vivo* imaging in this model (**Figure 1E**); however, the limitation is that the mice must be euthanized to quantify the metastatic tumor burden.

The purpose of the mastectomy model is to 1) reproduce the standard of care treatment of human breast cancer, which is the surgical removal of the primary tumor[17], and 2) allow the serial quantification of the metastatic tumor burden *in vivo*. This model is particularly useful in the preclinical study of the development of novel therapeutics[13]. As previously published, an Src inhibitor, AZD0530, which showed efficacy in the preclinical mice model but failed in a clinical trial for breast cancer treatment, showed efficacy in the primary lesion utilizing orthotopic inoculation without mastectomy but not in lung metastasis utilizing the mastectomy model presented here. Although anti-cancer drugs (*e.g.*, anthracyclines) are sometimes used in humans as neoadjuvant therapy to treat primary breast tumors, the vast majority of drugs are used as adjuvant therapy where drugs are given after surgery to reduce the risk of recurrence by treating clinically undetectable cancer or as palliative treatment for metastatic cancer[1,7,18]. Thus, this mastectomy model was established, which mimics the human treatment process[11].

Eight days after the cancer cell inoculation, the primary tumor is surgically removed (**Figure 2**). To test any of the drug efficacies for metastatic lesions, it should be administrated after the mastectomy. This model allows for lung metastatic lesion quantification, as well as whole-body metastatic lesion quantification, as long as the metastatic tumor burden has a detectable bioluminescence utilizing *in vivo* imaging. The anterior chest wall local recurrence rate is quite

low. In our experience, the local recurrence rate was less than 5% in over 50 mice experiments. However, if the postoperative day 1 bioluminescence exceeds 1.00E + 06 photons (10x larger than other individuals), there is a high possibility of a local residual tumor[13]. If there are remnant cancer cells present, a palpable tumor appears within two weeks. When there is a local residual tumor, the bioluminescence mainly reflects that local recurrence rather than any metastatic lesions. Local residual tumors are known to behave very differently than distant metastases[19]; thus, those animals with local recurrence (< 5% in our experience) should be excluded from any further analysis. This mastectomy model also allows for serial metastatic tumor burden monitoring without having to euthanize the animals. Furthermore, such bioluminescent monitoring can be confirmed by *ex vivo* lung metastasis quantification. Instead of quantifying lung metastases by *ex vivo* imaging, thus having to euthanize the animals, mice survival after surgical treatment can also be monitored as a translatable clinical endpoint (**Figure 2D**). Since there is no primary lesion, mice cannot meet euthanasia tumor criteria which are generally defined as a tumor size of > 2 cm or ulceration of the primary tumor. In this 4T1 mastectomy model, all mice died within 60 days after cancer cell inoculation due to metastasis.

**FIGURE LEGENDS:**

**Figure 1: Overview of the orthotopic model without mastectomy.** (**A**) This panel shows a time course of the orthotopic model utilizing the 4T1 syngeneic model. (**B**) This panel shows the whole-body bioluminescence of an orthotopic model which mainly reflects the primary tumor. The error bar indicates the standard error of the mean (*n* = 10). (**C**) This panel shows serial images of whole-body bioluminescence (of the same mouse). The scale bar indicates 1 cm. (**D**) This panel shows the actual tumor size of panel **B**, measured by calipers. The error bar indicates the standard error of the mean (*n* = 10). (**E**) This panel shows lung *ex vivo* bioluminescence images of 10 orthotopic model mice (*n* = 10). The scale bar indicates 1 cm.

**Figure 2: Overview of the mastectomy model.** (**A**) This panel shows a time course of the mastectomy model after 4T1 orthotopic syngeneic implantation. The scale bar indicates 1 cm. (**B**) This panel shows the whole-body bioluminescence of the mastectomy model, which mainly reflects metastatic lesion. The error bar indicates the standard error of the mean (*n* = 10). (**C**) This panel shows serial images of whole-body bioluminescence (of the same mouse). Utilizing *ex vivo* imaging, it was confirmed that the signals were from lung metastasis, and no local recurrence was confirmed by whole-body imaging after lung removal. The scale bar indicates 1 cm. (**D**) This panel shows the Kaplan-Meier survival curve of the mastectomy model without any drug treatment.

**DISCUSSION:**

For the last decade, we have been establishing multiple murine cancer models, including breast cancer models[3,7,13,16,20,21]. Previously, we demonstrated that breast cancer cell orthotopic inoculation into the mammary gland tissue under direct vision produced a larger tumor with less size variability compared to injecting cells around the nipple without a surgical incision[7]. This model has been improved upon by utilizing a cell suspension in a gelatinous protein mixture. The shapes of the tumor generated by the gelatinous protein mixture-suspended cells were round

with less variability, whereas those generated by PBS-suspended cells were scattered in the mammary gland[3].

We further verified that the tumors generated by the surgical orthotopic implantation method presented here highly expressed the genes whose interaction networks are important in cancer progression compared to those generated by non-surgical or subcutaneous injection[7]. These findings imply that this model mimics human breast cancer better compared to non-surgical orthotopic or subcutaneous implantation[7]. We also demonstrated that this model is suitable to evaluate the immune-mediated regression of breast cancer using allogenic rejection[3]. However, skin incision can also cause inflammation around the tumor[14]. Therefore, depending on the hypothesis tested by the investigator, it is important to consider inflammation as a possible confounding factor.

Other than the neoadjuvant setting, the vast majority of systemic therapies are administered after the primary breast tumor is surgically removed[11]. To date, the majority of preclinical studies for new drug development evaluate drug response in the primary tumor, but not in the metastatic lesions[1,18]. This discrepancy between animal models and human treatment is important because the gene profile of the metastatic lesions is significantly different from those of the primary lesion[22], which has important implications for cancer biology and treatment, especially in the era of targeted therapy[22]. Therefore, the efficacy of the drug for adjuvant therapy should be evaluated in the metastatic lesions, not merely in the primary tumor. We verified the timing of the lymph node and lung metastasis formation after orthotopic inoculation, utilizing the 4T1 breast cancer orthotopic model[16]. We also demonstrated that a resection of the primary tumor improved survival in the metastatic breast cancer utilizing the mastectomy model[23]. Thus, we tried to establish a mastectomy model after orthotopic cancer cell implantation. However, a long time was spent to establish this low local recurrence model. Because cancer cells are injected through surgical incision, cancer cells easily migrate into the wound. In addition, cancer cells invade tissues in direct contact with the tumor, such as the surrounding chest wall muscle and skin. The cancer cells also metastasize to axillary lymph nodes without forming a visible axillary mass at the time of the mastectomy. While removing the tumor may be an easier technique, doing so is not translatable to mastectomy in breast cancer treatment as it causes local recurrence[13]. Thus, this “radical mastectomy model” was established in order to remove all cancer cells from the anterior chest wall and axillary lymph node basin, as described in **Figure 2**. We confirmed lung metastasis by histology and lung *ex vivo* imaging, and there was no shining lesion present by whole-body imaging after lung removal. In this 4T1 orthotopic model, cancer cells eventually metastasize to the lungs, in all cases within 21 days. However, the timing of metastasis depends on the primary tumor size; thus, less primary tumor size variability is important for monitoring not only the primary tumor but also the metastatic tumor burden. By using the gelatinous protein mixture, tumor size variability can be minimized. However, the gelatinous protein mixture can also affect the tumor microenvironment. Therefore, the potential confounding factors associated with gelatinous protein mixture use should be considered, depending on the hypothesis of the investigator.

Utilizing these techniques and luciferase-expressing cancer cells allow for tumor burden quantification with less measurement error, even in non-palpable tumors. There are two reporter systems, bioluminescent and fluorescent, to visualize the signals in live animals. While it has been reported that the signal of both bioluminescence and fluorescence can be bright enough for quantification, when the target signal is very low, the background signal of fluorescence is higher, which reduces the accuracy of the assay. In contrast, bioluminescence is detected with greater accuracy at a lower signal with less background interference[24]. Accordingly, most investigators prefer to use bioluminescence reporter systems in live animal experiments[24,25]. However, tumor burden quantification by bioluminescence has its limitations. Luciferase-expressing cells shine when luciferin reaches the cancer cells by cardiovascular perfusion[26]. If the lesion has an area of hypoxia and/or hypoperfusion, luciferin is not delivered to the cells and the bioluminescence value could, then, be measured to be lower than the actual tumor burden. Indeed, we have experienced inappropriate bioluminescence quantification when the tumor size reached more than 1.5 cm. We assume that it is due to the described hypoxic and/or hypoperfused condition. Thus, when the tumor reaches a palpable size, the tumor burden is quantified by both bioluminescence and caliper measurement. An important limitation of bioluminescence quantification to consider is the black fur of mice. Even after the fur is removed, there is pigmentation on the skin[27]. While bioluminescence can be detected and quantified in mice with black fur, it is decreased and, therefore, it is important to compare mice of the same fur color to each other in order to reduce the confounding effects of fur color on bioluminescent quantification. The use of luciferase-expressing cells provides some unique modalities for study. For example, secretable luciferase can be used for tumor burden quantification by measuring blood or urine bioluminescence[28]. In the model presented here, direct tumor burden quantification of viable cancer cells in live animals with non-secretable luciferase is simple and straightforward, which does not require collecting blood or urine samples.

Similar to the models which introduced here, the production of a more efficient animal experiment by producing a higher success rate of tumorigenesis with less variability is key to prove the findings. In the present model, once an investigator has learned the technique, it is easy to achieve a 100% tumorigenesis rate. We also achieved higher tumorigenesis rates other than the models we have introduced; plus, we have established a colon cancer orthotopic model utilizing cell suspension in a gelatinous protein mixture, together with bioluminescence technology to quantify the tumor burden[21]. In conclusion, the animal model presented here provides a suitable murine model to conduct cancer study.

**ACKNOWLEDGMENTS:**

This work was supported by NIH grant R01CA160688 and Susan G. Komen Foundation Investigator Initiated Research grant (IIR12222224) to K.T. Mice bioluminescence images were acquired by shared resource Translational Imaging Shared Resource at Roswell Park Comprehensive Cancer Center, which was supported by the Cancer Center Support Grant (P30CA01656) and Shared Instrumentation grant (S10OD016450).

**DISCLOSURES:**

The authors have nothing to disclose.

**REFERENCES:**


1. Rashid, O. M., Takabe, K. Animal models for exploring the pharmacokinetics of breast cancer therapies. *Expert Opinion on Drug Metabolism & Toxicology.* **11** (2), 221-230 (2015).

2. Schuh, J. C. Trials, tribulations, and trends in tumor modeling in mice. *Toxicologic Pathology.* **32 Suppl 1**, 53-66 (2004).

3. Katsuta, E. *et al.* Modified breast cancer model for preclinical immunotherapy studies. *Journal of Surgical Research.* **204** (2), 467-474 (2016).

4. Sidell, D. R. *et al.* Composite mandibulectomy: a novel animal model. *Otolaryngology–Head and Neck Surgery.* **146** (6), 932-937 (2012).

5. Ewens, A., Mihich, E., Ehrke, M. J. Distant metastasis from subcutaneously grown E0771 medullary breast adenocarcinoma. *Anticancer Research.* **25** (6b), 3905-3915 (2005).

6. Kocaturk, B., Versteeg, H. H. Orthotopic injection of breast cancer cells into the mammary fat pad of mice to study tumor growth. *Journal of Visualized Experiments.* (96), e51967 (2015).

7. Rashid, O. M. *et al.* An improved syngeneic orthotopic murine model of human breast cancer progression. *Breast Cancer Research and Treatment.* **147** (3), 501-512 (2014).

8. Bertozzi, S. *et al.* Prevalence, risk factors, and prognosis of peritoneal metastasis from breast cancer. *SpringerPlus.* **4**, 688 (2015).

9. Kennecke, H. *et al.* Metastatic behavior of breast cancer subtypes. *Journal of Clinical Oncology.* **28** (20), 3271-3277 (2010).

10. Valero, M. G., Golshan, M. Management of the Axilla in Early Breast Cancer. *Cancer Treatment and Research.* **173**, 39-52 (2018).

11. National Comprehensive Cancer Network. *Breast Cancer, NCCN Clinical Practice Guidelines in Oncology*. https://www.nccn.org/professionals/physician_gls/pdf/breast.pdf (2018).

12. Versteeg, H. H. *et al.* Inhibition of tissue factor signaling suppresses tumor growth. *Blood.* **111** (1), 190-199 (2008).

13. Katsuta, E., Rashid, O. M., Takabe, K. Murine breast cancer mastectomy model that predicts patient outcomes for drug development. *Journal of Surgical Research.* **219**, 310-318 (2017).

14. Veenhof, A. A. *et al.* Surgical stress response and postoperative immune function after laparoscopy or open surgery with fast track or standard perioperative care: a randomized trial. *Annals of Surgery.* **255** (2), 216-221 (2012).

15. Wei, S., Siegal, G. P. Surviving at a distant site: The organotropism of metastatic breast cancer. *Seminars in Diagnostic Pathology.* **35** (2), 108-111 (2018).

16. Nagahashi, M. *et al.* Sphingosine-1-phosphate produced by sphingosine kinase 1 promotes breast cancer progression by stimulating angiogenesis and lymphangiogenesis. *Cancer Research.* **72** (3), 726-735 (2012).

17. Jones, C., Lancaster, R. Evolution of Operative Technique for Mastectomy. *Surgical Clinics of North America.* **98** (4), 835-844 (2018).

18. Rashid, O. M., Maurente, D., Takabe, K. A Systematic Approach to Preclinical Trials in Metastatic Breast Cancer. *Chemotherapy* (Los Angeles). **5** (3) (2016).

19. Ramaswamy, S., Ross, K. N., Lander, E. S., Golub, T. R. A molecular signature of metastasis in primary solid tumors. *Nature Genetics.* **33** (1), 49-54 (2003).

20. Aoki, H. *et al.* Murine model of long-term obstructive jaundice. *Journal of Surgical Research.* **206** (1), 118-125 (2016).

21. Terracina, K. P. *et al.* Development of a metastatic murine colon cancer model. *Journal of Surgical Research.* **199** (1), 106-114 (2015).

22. Rashid, O. M. *et al.* Is tail vein injection a relevant breast cancer lung metastasis model? *Journal of Thoracic Disease.* **5** (4), 385-392 (2013).

23. Rashid, O. M. *et al.* Resection of the primary tumor improves survival in metastatic breast cancer by reducing overall tumor burden. *Surgery.* **153** (6), 771-778 (2013).

24. Troy, T., Jekic-McMullen, D., Sambucetti, L., Rice, B. Quantitative comparison of the sensitivity of detection of fluorescent and bioluminescent reporters in animal models. *Molecular Imaging.* **3** (1), 9-23 (2004).

25. Adams Jr., S. T., Miller, S. C. Beyond D-luciferin: expanding the scope of bioluminescence imaging *in vivo*. *Current Opinion in Chemical Biology*. **21**, 112-120 (2014).

26. Close, D. M., Xu, T., Sayler, G. S., Ripp, S. In vivo bioluminescent imaging (BLI): noninvasive visualization and interrogation of biological processes in living animals. *Sensors* (Basel). **11** (1), 180-206 (2011).

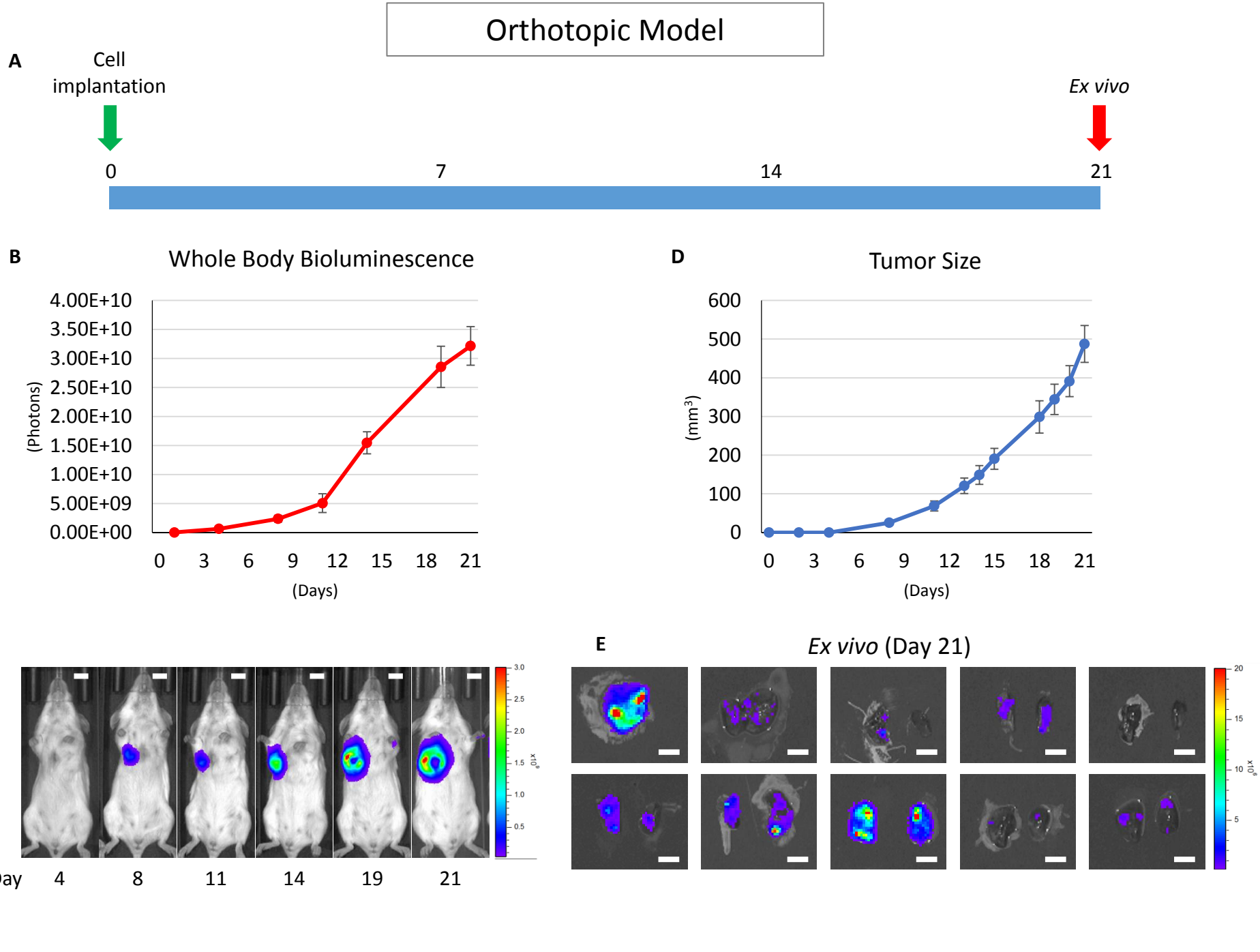
27. Chen, H., Thorne, S. H. Practical Methods for Molecular In Vivo Optical Imaging. *Current Protocols in Cytometry.* **59** (1224), 12.24.11-12.24.11 (2012).

28. Wurdinger, T. *et al.* A secreted luciferase for *ex vivo* monitoring of *in vivo* processes. *Nature Methods.* **5** (2), 171-173 (2008).

29. Aoki, H. *et al.* Host sphingosine kinase 1 worsens pancreatic cancer peritoneal carcinomatosis. *Journal of Surgical Research.* **205** (2), 510-517 (2016).

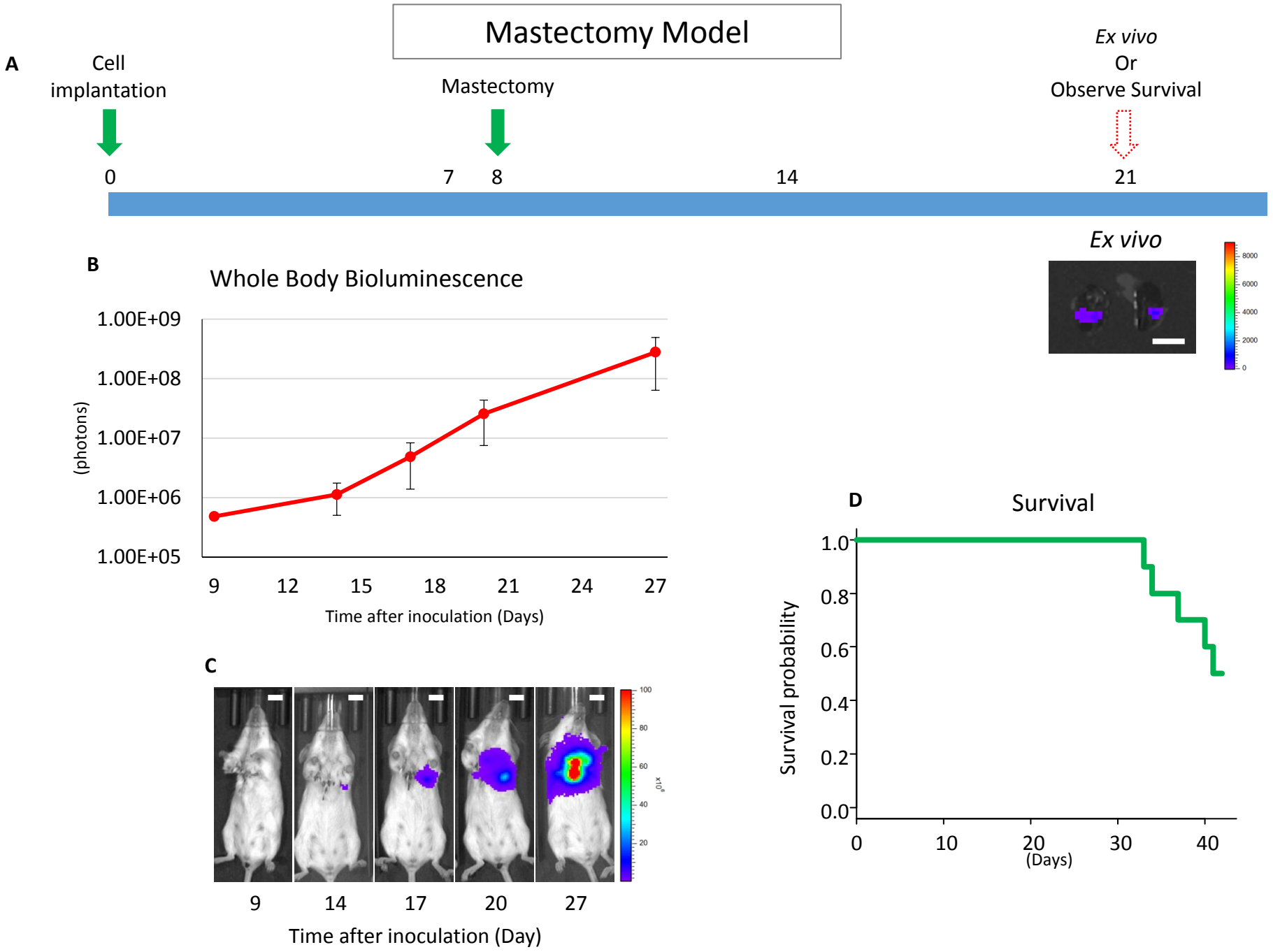
Figure

Click here to access/download;Figure;fig 1.pdf



Figure

Click here to access/download;Figure;fig 2.pdf



| Name of Reagent/ Equipment | Company | Catalog Number | Comments/Description |
|---|---|---|---|
| Micro Dissection Scissors | Roboz | RS-5983 | For cancer cell inoculation and masstectomy |
| Adson Forceps | Roboz | RS-5233 | For cancer cell inoculation and masstectomy |
| Needle Holder | Roboz | RS-7830 | For cancer cell inoculation and masstectomy |
| Mayo | Roboz | RS-6873 | For ex vivo |
| 5-0 silk sutures | Look | 774B | For cancer cell inoculation and masstectomy |
| Dry sterilant (Germinator 500) | Braintree Scientific | GER 5287-120V | For cancer cell inoculation and masstectomy |
| Clipper | Wahl | 9908-717 | For cancer cell inoculation and masstectomy |
| Matrigel | Corning | 354234 | For cancer cell inoculation |
| D-Luciferin, potassium salt | GOLD-Bio | LUCK-1K | For bioluminescence quantification |
| Roswell Park Memorial Insitute 1640 | Gibco | 11875093 | For cell culture |
| Fetal Bovine Serub | Gibco | 10437028 | For cell culture |
| Trypsin-EDTA (0.25%) | Gibco | 25200056 | For cell culture |

1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

# ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: How to generate murine orthotopic metastatic breast cancer model and perform murine radical mastectomy

Author(s): Eriko Katsuta, Kazuaki Takabe

Item 1 (check one box): The Author elects to have the Materials be made available (as described at http://www.jove.com/author) via: [x] Standard Access [ ] Open Access

Item 2 (check one box):

[x] The Author is NOT a United States government employee.

[ ] The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

[ ] The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

## ARTICLE AND VIDEO LICENSE AGREEMENT

1. Defined Terms. As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of *The Journal of Visualized Experiments;* "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. Background. The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. Grant of Rights in Article. In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. Retention of Rights in Article. Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. Grant of Rights in Video – Standard Access. This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. Grant of Rights in Video – Open Access. This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this Section 6 is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. Government Employees. If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. Likeness, Privacy, Personality. The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

9. Author Warranties. The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

10. JoVE Discretion. If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have

full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

11. Indemnification. The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in *JoVE* or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

12. Fees. To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

13. Transfer, Governing Law. This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement required per submission.

**CORRESPONDING AUTHOR:**

| | |
|---|---|
| Name: | Kazuaki Takabe |
| Department: | Surgical Oncology |
| Institution: | Roswell Park Cancer Institute |
| Article Title: | How to generate murine orthotopic metastatic breast cancer model and perform murine radical mastectomy |
| Signature: |  Date: 1/3/2018 |

Please submit a signed and dated copy of this license by one of the following three methods:

1) Upload a scanned copy of the document as a pfd on the JoVE submission site;
2) Fax the document to +1.866.381.2236;
3) Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02139

For questions, please email submissions@jove.com or call +1.617.945.9051

## Author's Point-by-point Response the Editorial Comments

## Manuscript #57849_R1

**Editorial comments:**

**Comment A1:** *The manuscript will benefit from thorough language revision as there are a number of grammatical errors throughout. Please thoroughly review the manuscript and edit any errors. Several sentences are poorly structured.*

**Response A1:** We appreciate the Editorial comment. The manuscript was reviewed and edited by a native English speaker who is proficient in scientific writing.

**Comment A2:** *How was this compared? Do you report this herein? Please do not make claims that's are unsupported. This model does not mimic human breast cancer progression, but attempts to mimic it.*

**Response A2:** As per the Editorial comments, we revised the short abstract as below.

**"We introduced a murine orthotopic breast cancer model and radical mastectomy model with bioluminescence technology to quantify tumor burden in which we sought to mimic human breast cancer progression."**

**Comment A3:** *"Luciferase-expressing" may be a better fit.*

**Response A3:** As per the Editorial comments, we revised as **"Luciferase-expressing".**

**Comment A4:** *Please expand your Introduction to include the following:*

**Comment A4-1:** *The advantages over alternative techniques with applicable references to previous studies (avoid self citations);*

**Response A4-1:** As per the Editorial comment, we added the sentenced in the Introduction section as below.

**“To mimic human breast cancer progression, we chose the #2 mammary fat pad as an inoculation site which is located in the chest. In most of the studies, breast cancer cells are inoculated subcutaneously. This technique does not require surgery and thus it is simple and straightforward. However, the subcutaneous microenvironment is quite different than the mammary grand microenvironment, which therefore results in a different pattern of cancer progression and even molecular profiles compared to mammary gland inoculated tumors. Some studies use the #4 mammary gland, which is located in the abdomen, as an inoculation site. However, because the #4 mammary glands are located on the abdomen, the most common metastatic pattern is peritoneal carcinomatosis, which is stark contrast to human breast cancer progress where peritoneal carcinomatosis occurs in less than 10% of metastatic breast cancer cases. Breast cancer generated by our technique in the #2 mammary gland metastasizes to the lung which is one of the most common human breast cancer metastatic sites.”**

**“Cancer cell inoculation via the tail vein is the most common lung metastasis mouse model, the so called “experimental metastasis”. This model is easy to generate and does not require surgery; however, it does not mimic human breast cancer progression which may result in different metastatic disease behavior. In order to mimic the human breast cancer treatment course where metastasis often occurs after mastectomy, we removed the primary tumor after orthotopic cancer cell inoculation.”**

**Comment A4-2:** *Description of the context of the technique in the wider body of literature;*

**Response A4-2:** As per the Editorial comment, we revised the sentenced in the Introduction section as below.

**“Breast cancer generated by our technique in the #2 mammary gland metastasizes to the lung which is one of the most common breast cancer metastatic sites. In this technique, we also sought to achieve a higher tumorigenesis rate with minimal tumor size variability. To do so, cancer cells suspended in a gelatinous protein mixture were inoculated under direct vision through a median anterior chest wall incision. This technique produced a high tumorigenesis rate with less variability in tumor size and shape compared to subcutaneous or non-surgical injection.”**

**“We also introduced a mouse radical mastectomy technique in which the orthotopic breast tumor is resected with surrounding tissues and axillary lymph nodes. Cancer cell inoculation via tail vein is the most common lung metastasis mice model, the so called “experimental metastasis”. This model is easy to generate and does not require surgery; however, it does not mimic human breast cancer progression which may result in different metastatic disease behavior. Thus, we removed the primary tumor after orthotopic cancer cell inoculation which mimics human breast cancer treatment course.”**

**Comment A4-3:** *Information that can help readers to determine if the method is appropriate for their application.*

**Response A4-3:** This model is applicable to most cases, thus we stated as below.

**"The techniques we described are applicable for most of breast cancer orthotopic model experiments. However, it is important to consider that the gelatinous protein mixture can affect the microenvironment and surgery can affect the stress/immune response."**

**Comment A5:** *References?*

**Response A5:** As per the Editorial comment, we added the citation.

**Comment A6:** *References?*

**Response A6:** As per the Editorial comment, we added the citation.

**Comment A7:** *References for the well established approaches?*

**Response A7:** As per the Editorial comment, we added the citation.

**Comment A8:** *References for the well established approaches? A description of the standard mastectomy approach in humans will help here.*

**Response A8:** As per the Editorial comment, we added the sentences in the Introduction section as below.

**"In the clinical setting, the standard of care for breast cancer patients without distant metastasis disease is mastectomy. Before mastectomy, axillary lymph node metastasis is surveyed by imaging and sentinel lymph mode biopsy. If there is no evidence of axillary lymph node metastasis, the patient is then treated by total or partial mastectomy, in which the axillary lymph node resection is omitted. Total mastectomy is a technique to resect breast cancer with the whole breast tissue en bloc, whereas partial mastectomy is to resect breast cancer with a margin of surrounding normal**

**breast tissue only, thus conserving the remaining normal breast tissue in the patient. However, patients who preserve their remaining normal breast tissue after partial mastectomy require postoperative radiotherapy to avoid local recurrence. Patients who have axillary lymph node metastasis undertake radical mastectomy which removes the breast cancer with all normal breast tissue and axillary lymph nodes and invaded tissues en bloc. In the mouse model, surveillance for axillary lymph node metastasis and/or post-operative radiation is not reasonable or feasible. Thus, we utilized the radical mastectomy technique to avoid local recurrence."**

**Comment A9:** *Higher success rate as compared to what?*

**Response A9:** As per the Editorial comment, we added the sentences in the Introduction section as below.

**"The aims of these methods are 1) to mimic human breast cancer progression and treatment course, 2) to conduct in vivo experiments with greater efficiency and higher success rates compared to other breast cancer inoculation or mastectomy techniques."**

**Comment A10:** *Improve compare to what? If previous work, then references must be cited.*

**Comment A11:** *Higher than?*

**Response A10, A11:** As per the Editorial comment, we revised the sentences in the Introduction section as below.

**"In this technique, we also sought to achieve a higher tumorigenesis rate with minimal tumor size variability compared to other breast cancer inoculation techniques."**

**Comment A12:** *Was this quantified later on? Unclear how you can claim this without a thorough analysis.*

**Response A12:** We apologize for unclear statement. It was demonstrated by our previous work. We added the sentences in the Introduction section as below.

**"This technique produced a high tumorigenesis rate with less variability in tumor size and shape compared to subcutaneous or non-surgical injection, as we have previously reported."**

**Comment A13:** *Axillary?*

**Response A13:** We apologize for the error in the spelling. It was revised as **"axillary".**

**Comment A14:** *Was this quantified later on? Unclear how you can claim this without a thorough analysis*

**Response A14:** We apologize for the unclear statement. It was demonstrated by our previous work. We revised the sentence in the Introduction section as below.

**"This technique produced less local recurrence compared to simple tumor resection, as we have previously reported,"**

**Comment A15:** *Needs references.*

**Response A15:** As per the Editorial comment, we added the reference.

**Comment A16:** *Please add a step to briefly describe how the cells are cultured and maintained. Please include medium used, incubation environmental conditions etc.*

**Response A16:** As per the Editorial comment, we added 2.1) as below.

**"2.1) Culture 4T1-luc2 cells, a mouse mammary adenocarcinoma cell line expressing luciferase, in Roswell Park Memorial Institute 1640 media with 10% fetal bovine serum in a humidified incubator at 37°C in 5% $CO_2$."**

**Comment A17:** *Do they adhere to the dish?*

**Response A17:** As per the Editorial comment, we revised the sentence of 2.2) as below.

**"2.2) Wash adherent 4T1-luc2 cells in a 10 cm dish with phosphate-buffered saline (PBS)"**

**Comment A18:** *Please change "degrees" to " °C" throughout.*

**Response A18:** As per the Editorial comment, we revised "degree" to **"°C"** throughout.

**Comment A19:** *What is the composition? Please add it to the table of materials.*

**Response A19:** As per the Editorial comment, we revised 2.2) as below and added materials in the table of materials.

**"Then, add 4ml growth media (RPMI-1640 with 10% FBS)"**

**Comment A20:** *Transfer the cell suspension? unclear*

**Response A20:** As per the Editorial comment, we revised 2.2) as below.

**"transfer the cell suspension into a 15 mL conical tube"**

**Comment A21:** *Please convert to g.*

**Response A21:** As per the Editorial comment, we revised "1500 rpm" to **"180 x g".**

**Comment A22:** *Please revise for grammar.*

**Response A22:** As per the Editorial comment, we revised the sentence as below.

**"we use $1x10^4$/20 µL of final concentration, to avoid reaching euthanasia criteria (tumor size > 2cm) within 2 weeks."**

**Comment A23:** *Mention oxygen flow rate.*

**Response A23:** As per the Editorial comment, we added about oxygen flow as below.

**"3.1) Put the mice in the anesthesia induction chamber with 2.5% isoflurane and 0.2 L/min oxygen flow until the mice breathe calmly (2-3 min)."**

**Comment A24:** *Please mention how you ensure depth of anesthesia (e.g. toe pinch).*

**Response A24:** As per the Editorial comment, we added about oxygen flow as below.

**"3.3) Confirm adequate anesthesia by the lack of reaction to toe pinch."**

**Comment A25:** *Limbs?*

**Response A25:** As per the Editorial comment, we revised "legs and arms" to **"limbs".**

**Comment A26:** *Are analgesics provided? Mention dosage.*

**Response A26:** Analgesics was not required, but we anesthetized the mice by isoflurane. We revised the sentence as below.

**"3.9) Remove the sutures under anesthesia same as 3.1) 7 days after surgery."**

**Comment A27:** *In how many animals did you observe this? I'm also not sure about what purpose this statement serves here. Is this typical for this model? Is this reported by others for this particular cell line? Please provide references. This seems incredibly early for the number of cells injected. How did you confirm the presence of lung metastasis without lung resection and imaging? How do you know that the injected cells were not localized in the lungs rather than a true metastasis?*

**Response A27:** The purpose of this statement is to illustrate that timing of lung metastasis is varied by cell line or injected cell number. We confirmed that 100% of mice in over 50 mice experiments developed lung metastasis at 8 days after inoculation. Thus, we described it here to show the timing of lung metastasis in this particular setting. To clarify these points, we revised the sentence as below.

**"Note: Timing of mastectomy is very important. If it is too early, lung metastasis does not occur. If it is too late, the primary tumor has invaded major blood vessels which make complete oncologic resection challenging. Thus, we tested multiple time points for mastectomy to determine which time**

**point produced the appropriate balance in waiting for metastasis before resection became too challenging. After doing so in over 50 mouse experiments it was demonstrated that mastectomy at 8 days after cancer cell inoculation (or when tumor size reaches 5 mm) was the ideal time point to achieve that balance[13].”**

**Comment A28:** *Unclear, needs grammar revision*

**Response A28:** As per the Editorial comment, we revised the sentence as below.

**“Make a 5 mm skin incision in 2 mm left from the surgical scar which was made at the initial cancer cell inoculation, using the micro dissection scissors.”**

**Comment A29:** *Forelimb?*

**Response A29:** As per the Editorial comment, we revised “arm” to **“forelimb”.**

**Comment A30:** *How many? How do you identify them? Can you provide a photograph to aid identification?*

**Response A30:** We apologize for the unclear statement. Most of the time, the axillary lymph nodes are not visible at the time of mastectomy; however, en bloc resection with axillary lymph nodes is important to avoid visible recurrence by bioluminescence in order to monitor lung metastasis. To clarify these points, we revised the sentence as below and added the citation of our previous publication which contains the picture of the mastectomy.

**“Extend the incision toward the root of the forelimb to remove the tumor, the skin including the surgical scar and the lesion in contact with the tumor as well as the axillary lymph node basin in which most of the time no visible lymph node exist at the time of mastectomy[13].”**

**Comment A31:** *Are analgesics provided? Mention dosage.*

**Response A31:** Analgesics was not required, but we did anesthetize the mice by isoflurane. We revised the sentence as below.

**“4.6) Remove the sutures under anesthesia same as 3.1) 7 days after surgery.”**

**Comment A32:** *Shouldn’t this section be placed before section 4? Or do you image the animal after mastectomy?*

**Comment A33***At what time points after inoculation?*

**Response A32, 33:** We apologize for the confusing title and lack of adequate explanation. Bioluminescence can be used for both primary tumor (without mastectomy) and lung metastasis (mastectomy model) quantification regularly. To clarify these points, we revised the title and added the note as below.

**“5. Bioluminescent quantification of the primary tumor (orthotopic inoculation without mastectomy) or lung metastasis (mastectomy model)”**

**“Note: For primary tumor burden quantification, the bioluminescence is measured twice a week from the day after orthotopic inoculation. For lung metastasis quantification, the bioluminescence is measured twice a week from the day after mastectomy.”**

**Comment A34:** *Degree Celsius?*

**Response A34:** As per the Editorial comment, we revised “degrees” to **“°C”.**

**Comment A35:** *In prone position?*

**Response A35:** The mouse is placed in the supine position. We revised the sentence as below.

**“5.6) Fit each mouse with a nose cone inside the imaging system in the supine position”**

**Comment A36:** *This seems pretty long? Wouldn’t Luciferin clear out by this*

**Response A36:** Peak times varied by individuals and by the day of image acquisition; however, sometimes it takes around 50 minutes to reach peak bioluminescence. We have no experience of problems

due to luciferin clear out. In our technique, cells were suspended in 90% Matrigel. We think due to a high concentration of Matrigel, it takes a long time for luciferin delivery to cancer cells.

**Comment A37:** *What is the acquisition duration per image?*

**Response A37:** It depends on exposure time. As we described in 5.7.1), we usually use "auto" exposure time which the software automatically chooses as the suitable exposure time. It is decided by the signal intensity of the original cell and the size of tumor burden, in another words, total luciferase-luciferin shining amount.

**Comment A38:** *By ex vivo what? Sound incomplete.*

**Response A38:** As per the Editorial comment, we revised the title as below.

**"6. Lung metastasis tumor burden quantification by ex vivo imaging"**

**Comment A39:** *Why was this time point chosen? Also, is this done on the animals that have undergone mastectomy?*

**Response A39:** As per the Editorial comment, we added "Note" as below.

**"Note: lung metastasis quantification is applicable for both orthotopic inoculation with and without mastectomy models. In the mastectomy model, ex vivo imaging or survival observation is chosen depending on the purpose. In the orthotopic inoculation (without mastectomy) model, most cases produce primary tumor size euthanasia criteria (2.0 cm) approximately 21 days after inoculation."**

**Comment A40:** *By ex vivo what? This is incomplete. Do you mean "Ex vivo imaging"?*

**Response A40:** We apologize for the error. As per the Editorial comment, we revised as **"ex vivo imaging".**

**Comment A41:** *Luciferase image? Mention acquisition settings?*

**Response A41:** As per the Editorial comment, we revised the sentence as below.

**"6.12) Capture the bioluminescence image (same as 5.7.1) and 5.7.2)) 5 min after euthanasia (20 min after luciferin injection)."**

**Comment A42:** *This progression is not always follows. Lung metastasis can happen by the lymphatic and/or blood route, and there is likely no sequence as such where the lung metastasis follows the nodal metastasis. Please cite a reference to back this up.*

**Response A42:** As per the Editorial comment, we added the citation.

**Comment A43:** *Provide the equation used for this or a reference for tumor volume measurements using calipers.*

**Response A43:** As per the Editorial comment, we added the sentence as below.

**"The tumor volume was estimated using the following equation: volume = (length) x $(width)^2/2$."**

**Comment A44:** *This is vague, unclear what is meant by this. Do you mean that they show similar trends?*

**Response A44:** We apologize for unclear statement. We revised the sentence as below.

**"Quantification of the tumor burden by bioluminescence and caliper measurement showed similar trends in the representative results".**

**Comment A45:** *Imaging?*

**Response A45:** We apologize for the error of the grammar. We revised as **"ex vivo imaging".**

**Comment A46:** *Please provide references for standard mastectomy.*

**Response A46:** As per the Editorial comment, we added citation for standard mastectomy.

**Comment A47:** *How can you be confident that all the luminescence signal you measure is coming from mets? Could it not be residual tumor that was not resected?*

**Response A47:** As we described in the Representative results section as below, if local recurrence occurs, it can be easily distinguished from the lung metastasis.

**"if the post-operative day one bioluminescence exceeds 1.00E + 06 photons (10 times larger than other individuals), there is a high possibility of local residual tumor[13]. If there are remnant cancer cells present, a palpable tumor appears within 2 weeks."**

**Comment A48/A49:** *Examples?*

**Response A48/A49:** We apologize for the unclear description for that. We revised the sentence as below.

**"This model is particularly useful in the preclinical study of the development of novel therapeutics[13]. As we have previously published, a Src inhibitor, AZD0530, which showed efficacy in preclinical mice model using but failed in clinical trial for breast cancer treatment, showed efficacy in the primary lesion utilizing orthotopic inoculation without mastectomy but not in lung metastasis utilizing our mastectomy model. Although anti-cancer drugs, e.g. anthracyclines, are sometimes used in humans as neoadjuvant therapy to treat primary breast tumors, the vast majority of drugs are used as adjuvant therapy where drugs are given after surgery to reduce the risk of recurrence by treating clinically undetectable cancer or as palliative treatment for metastatic cancer[1,7,18]."**

**Comment A50:** *Reference?.*

**Response A50:** As per the Editorial comment, we added the citation.

**Comment A51:** *Which drug? What is the dosage. This appear out of the blue and distracts the reader.*

**Response A51:** We apologize for the confusing description. We revised the sentence as below.

**“To test any of drug efficacies for metastatic lesions, it should be administrated after mastectomy.”**

**Comment A52:** *Ex vivo?*

**Comment A53:** *How was this done? In vivo or ex vivo? If in vivo, do you study the minimum met size for detection, and if ex vivo, do you extract all organs and perform imaging?*

**Response A52/53:** We apologize for the unclear description. We revised the sentences as below.

**“This model allows for lung metastatic lesion quantification as well as whole body metastatic lesion quantification as long as metastatic tumor burden has a detectable bioluminescence utilizing in vivo imaging.”**

**Comment A54:** *Please report the percentage recurrence*

**Response A54:** As per the Editorial comment, we added the sentence as below.

**“In our hands, local recurrence rate was less than 5% in over 50 mice experiments.”**

**Comment A55:** *References?*

**Response A55:** As per the Editorial comment, we added citation.

**Comment A56:** *What was the percentage of such cases in your study?*

**Comment A57:** *Is this for all the animals? Did you exclude animals with local recurrence?*

**Comment A58:** *What about the local recurrence?*

**Comment A60:** *What about the local recurrence?*

**Response A56/A57/A58/A60:** The percentage of the local recurrence cases is less than 5% and these animals were excluded from any of further analysis. To clarify this point, we revised the sentence as below.

**“thus those animals with local resurrence (<5% in our experience) should be excluded from any of further analysis.”**

**Comment A59:** *Please report the criteria*

**Response A59:** As per the Editorial comment, we revised the sentence as below.

**“Since there is no primary lesion, mice cannot meet euthanasia tumor criteria which are generally defined as tumor size > 2.0 cm or ulceration of the primary tumor.”**

**Comment A61/A62:** *Please report the sample sizes, how many animals in total?*

**Response A61/A62:** As per the Editorial comment, we added mice number.

**Comment A63:** *Is the number of days report, days after inoculation or days after mastectomy?*

**Response A63:** As per the Editorial comment, we revised x axis title as **“Time after inoculation (Days)”.**

**Comment A64:** *Is the number of days report, days after inoculation or days after mastectomy? This image strongly suggests that there was local recurrence and that the signal you see is not only metastasis. How do you claim that the signal in B is from mainly metastatic lesions? I do not believe this was decoupled here.*

**Response A64:** As per the Editorial comment, we revised the x axis title as **“Time after inoculation (Days)”.** And as we described, once local recurrence occurred, it grows very fast and signal intensity is much higher and reaches palpable size within 2 weeks. We confirmed that the signal was from the lung by lung ex vivo imaging and whole body imaging after lung removal. To clarify this point, we added the sentence as below.

**“It was confirmed that signals were from lung metastasis utilizing ex vivo imaging, and no local recurrence was confirmed by whole body imaging after lung removal.”**

**Comment A65:** *You mean no neoadjuvant or adjuvant treatment correct? Mastectomy is a form of treatment.*

**Response A65:** As per the Editorial comment, we revised the sentence as below.

**"Kaplan-Meier survival curve of mastectomy model without any of drug treatment."**

**Comment A66:** *References?*

**Comment A67:** *This needs references.*

**Comment A68:** *Needs references.*

**Comment A69:** *References?*

**Comment A70:** *References?*

**Comment A71:** *References?*

**Response A66/A67/A68/A69/A70/A71:** As per the Editorial comment, we added references.

**Comment A72:** *How do you know that the lung mets are metastasis and not cancer spread from contact (as you mention here)?*

**Response A72:** We confirmed lung metastasis by histology and lung ex vivo imaging, and also confirmed no shining lesion present by whole body imaging after lung removal. To clarify it, we added the sentence as below.

**"We confirmed lung metastasis by histology and lung ex vivo imaging, and we also confirmed no shining lesion present by whole body imaging after lung removal."**

**Comment A73:** *In how many days?*

**Response A73:** As per the Editorial comment, we revised the sentence as below.

**"In our 4T1 orthotopic model, cancer cells eventually metastasize to the lungs in all cases within 21 days."**

**Comment A74:** *Please reference other works where this is shown. My understanding is this is very common, but it is important to cite references to published literature.*

**Comment A75:** *References?*

**Response A74/A75:** As per the Editorial comment, we added references.

**Comment A76:** *Even if they are shaved?*

**Response A76:** Yes, even after they are shaved, there is a pigmentation issue. To clarify this point, we added the sentence as below.

**"Even after the fur is removed, there is pigmentation on the skin[27]."**

**Comment A77:** *What is your success rate so far? Please report this.*

**Response A77:** Once an investigator is getting used to this technique, it is easy to achieve 100% success rate. To clarify this point, we added the sentence as below.

**"In our model which we introduced here, once an investigator has learned this technique, it is easy to achieve a 100% tumorigenesis rate."**

**Comment A78:** *How do you quantify and estimate this?*

**Response A78:** It is largely dependent on the model. In our case, we compared tumorigenesis rate tumor size variability in multiple settings (subcutaneous inoculation, orthotopic inoculation without surgical incision and our model) in previously published work, as described in the Discussion section.

**Comment A79:** *How is this relevant here? Seems like you are just trying to cite more of your work in this paper*

**Response A79:** As per the Editorial comment, we removed the sentence.

**Comment A80:** *All of the references are self citations. Please include references to work from other groups and minimize the self citations.*

**Response A80:** Since we added more citations, now less than half the bibliography is composed of publications from our group.