Video Article

Quantitation of Intra-peritoneal Ovarian Cancer Metastasis

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Abstract

Epithelial ovarian cancer (EOC) is the leading cause of death from gynecologic malignancy in the United States. Mortality is due to diagnosis of 75% of women with late stage disease, when metastasis is already present. EOC is characterized by diffuse and widely disseminated intra-peritoneal metastasis. Cells shed from the primary tumor anchor in the mesothelium that lines the peritoneal cavity as well as in the omentum, resulting in multi-focal metastasis, often in the presence of peritoneal ascites. Efforts in our laboratory are directed at a more detailed understanding of factors that regulate EOC metastatic success. However, quantifying metastatic tumor burden represents a significant technical challenge due to the large number, small size and broad distribution of lesions throughout the peritoneum. Herein we describe a method for analysis of EOC metastasis using cells labeled with red fluorescent protein (RFP) coupled with *in vivo* multispectral imaging. Following intra-peritoneal injection of RFP-labelled tumor cells, mice are imaged weekly until time of sacrifice. At this time, the peritoneal cavity is surgically exposed and organs are imaged *in situ*. Dissected organs are then placed on a labeled transparent template and imaged *ex vivo*. Removal of tissue auto-fluorescence during image processing using multispectral unmixing enables accurate quantitation of relative tumor burden. This method has utility in a variety of applications including therapeutic studies to evaluate compounds that may inhibit metastasis and thereby improve overall survival.

Video Link

The video component of this article can be found at https://www.jove.com/video/53316/

Introduction

Epithelial ovarian cancer (EOC) is the most common cause of death from gynecologic malignancy, with an estimated 21,290 new diagnoses in the U.S. in 2015 and an estimated 14,180 deaths[1]. The vast majority (> 75%) of women are diagnosed with late-stage disease (stage III or IV) characterized by diffuse intra-peritoneal metastasis and poor prognosis. Disease recurrence in the peritoneal cavity following first-line chemotherapy is also common and represents a major cause of mortality[2,3]. EOC metastasizes by an unique mechanism involving both direct extension from the primary tumor to neighboring peritoneal organs as well as by dissociation or shedding of cells from the primary tumor surface as single cells or multi-cellular aggregates. Cells are shed into the peritoneal cavity, wherein they resist detachment-induced apoptosis[4]. Buildup of peritoneal ascites is common, as shed tumor cells block peritoneal lymphatic drainage and tumors produce growth factors that alter vascular permeability. A portion of shed tumor cells attach to the surface of peritoneal organs and structures including intestine, liver, omentum and mesentery, whereupon they anchor and proliferate to produce multiple widely disseminated secondary lesions[3,5]. Hematogenous metastasis is uncommon. Thus, clinical management commonly consists of cytoreductive surgery including "optimal debulking", defined as resection of all visible tumor (no matter how small). Complete cytoreduction is associated with a significant increase in overall survival[6,7] and is associated with the challenge of identification and removal of lesions < 0.5 cm.

Small animal models have proven utility in ovarian cancer research in improving our understanding of disease progression as well in identification of prognostic biomarkers and testing of novel chemotherapies or combination therapy approaches. As the primary site of ovarian cancer incidence and metastasis is the peritoneal cavity, orthotopic models of EOC metastasis involve the analysis and characterization of intraperitoneal disease. Although there have been recent improvements in the ability to image tumor cells, even at the single cell level, there still exist significant difficulties in quantifying the metastatic tumor burden of EOC. These challenges arise due to the number, size and anatomic location of metastatic lesions. Furthermore there exists a need to label cancer cells to distinguish them from normal host cells. Previous studies have utilized antibody-based labeling protocols or transfection of tumor cells with luciferase[8,9]. Direct fluorescent labeling of cancer cells was first reported by Chishima and coworkers in 1997[10]. Fluorescent labels do not require addition of exogenous substrate and provide exquisite tumor cell specificity, providing a more effective means to track cancer metastasis[11,12].

Herein we describe an optical imaging method for quantitative analysis of metastatic disease using a syngeneic orthotopic xenograft model comprised of red fluorescent protein (RFP)-tagged murine ID8 ovarian cancer cells[13] and immuno-competent C57/Bl6 mice. We demonstrate a novel method of relative tumor burden quantification combining *in vivo* and *ex vivo* imaging with removal of tissue auto-fluorescence. This

approach has potential utility in studies designed to evaluate the effect of specific genetic, epigenetic or micro-environmental modifications and/or treatment modalities on organ-specific metastasis of ovarian cancer.

Protocol

All *in vivo* studies were approved by the University of Notre Dame Animal Care and Use Committee and used female C57/BL6J mice.

1. Murine Ovarian Cancer Cell Culture

1. Make the ID8 murine ovarian cancer cell culture medium as follows: 1 L of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 4% Fetal Bovine Serum (FBS), 1% Penicillin/Streptomycin, 5 µg/ml Insulin, 5 µg/ml Transferrin and 5 ng/ml Sodium Selenite.
2. Transduce ID8 murine ovarian cancer cells[13] to express Red Fluorescent Protein (RFP) using commercially procured lentiviral particles expressing RFP and a selection marker (blasticidin gene). Note that green fluorescent protein can be used, but provides a weaker fluorescence signal.
    1. Immediately prior to transduction, culture cells to 50 - 70% confluence and add fresh medium (without penicillin/streptomycin) that includes 1 ml polybrene (5 µg/ml final concentration).
    2. Gently pipette RFP-lentivirus (20 µl) dropwise into the dish and incubate at 37 ^{o}C for 72 hr. Remove medium and incubate with fresh medium for 24 hr. Begin selection of transduced cells by adding blasticidin to the culture medium (5 µg/ml) and monitor for red cells using fluorescence microscopy (**Figure1**).
3. Culture the RFP-tagged cells in ID8 medium by seeding approximately 10^6 cells into a tissue culture dish and visualizing daily using a light microscope until the cell monolayer is confluent.
4. When confluent, harvest the cells using trypsin (0.25%, 3 min) and return the flask to the 37 ^{o}C incubator until the cells are detached.
5. Neutralize the trypsin with 6 ml of ID8 culture medium containing serum. Pipette up and down against the bottom of the dish, then transfer to a 15 ml conical tube. Centrifuge at 1,200 RPM for 2 min, then aspirate the medium using a glass pipette.
6. Wash the cells with Phosphate Buffered Saline (PBS) by gently resuspending in 6 ml warm PBS and centrifuging as in 1.5 above. Re-suspend in PBS to a final concentration of 5 x 10^6 cells/ml. Keep cells warm (37 ^{o}C) until injection.

2. Intra-peritoneal Injection of ID8 Cells and *in vivo* Imaging

1. Inject each mouse with 2 mL of the warm ID8-PBS solution for a total of 10 million cells through intra-peritoneal (i.p.) injection. Allow tumor cells to grow *in vivo* for approximately 10 weeks with weekly live imaging.
2. Immediately following injection and each week thereafter, weigh the mice and then anesthetize each mouse using isofluorane (2.5% flow rate in 0.5 L/min O_2 flow). Note: The mice were weighed, not as a quantitative measure of tumor burden, but rather a more general measure of individual mouse physical progression throughout the study. Mice weights were compared with their own previous weight values.
    1. Anesthetize mice by placing in an isofluorane chamber. Maintain constant anesthesia while scanning by positioning the mouse head in the nose-cone for exposure to 2.5% flow rate isofluorane throughout the procedure.
3. Use depilatory cream to remove the hair on the torso and abdomen of each mouse, as the black hair will cause attenuation of the fluorescence signal. Mice are bathed with warm water after application of the depilatory cream and dried with RT paper towels.
4. While anesthetized, scan the entire body of each mouse using a small animal optical imaging system. Scan all mice in a cohort at each time point. Use the following **two step protocol** (**Figure 2A**):
    1. In Step 1, to observe fluorescently labeled (RFP) tumor cells, execute multispectral acquisition to collect a total of 5 images using excitation filters of 440 nm, 460 nm, 480 nm, 520 nm and 540 nm, and an emission filter of 600 nm. Use the following acquisition parameters: a standard exposure of 15 sec, 2 x 2 binning, FOV of 160 mm, and f_{stop} of 1.1.
    2. In Step 2, to observe the whole animal, acquire a reflectance image using an open excitation filter (white light), an open emission filter, a standard exposure of 0.2 sec with 2 x 2 binning and an FOV of 16 cm. Note: The actual imaging time is ~1 min.

3. Mouse Dissection and Image Acquisition

1. When live imaging shows the presence of extensive intra-peritoneal disease or animals exhibit accumulation of ascites (as evidenced by abdominal distension), loss or gain of body weight greater than 20%, lethargy or other signs of distress, euthanize each mouse using CO_2 anesthesia followed by cervical dislocation.
2. Cut the skin anteriorly along the ventral midline of the mouse, using pointed dissection scissors, from the top of the thighs to the bottom of the rib cage. Take extreme care to cut through only the skin layer (**Figure 2Bi**). Gently separate the skin from the peritoneal tissue, being sure to not puncture the peritoneal wall.
3. Using pointed dissection scissors, cut laterally both along the bottom of the rib cage and the top of the thighs to create two flaps of skin (**Figure 2B ii,iii**).
4. Place each mouse on its ventral side (peritoneal cavity facing downward) with the skin flaps pulled open and scan each mouse with its organs *in situ* using the small animal optical imaging system (**Figure 3A,B**).
    1. Use the same image acquisition parameters as described in 2.4.1.-2.4.2.
5. Continue dissecting the mouse by extracting individual peritoneal organs including liver, omentum/pancreas, stomach, diaphragm, small intestine, large intestine, left and right peritoneum, ovaries, kidneys, mesentery and fat pad.
    1. Observe, enumerate, and record visible tumors on each organ. Use a caliper to measure the diameter of each tumor.
    2. Designate tumors less than **2 mm** in diameter as **small**, in between **2** and **5 mm** as **medium** and greater than **5 mm** as **large**.

6. Place the organs on the **organ scanning template** (**Figure 4**) that identifies a predetermined location for each organ. Use PBS to keep the organs moist.
7. Scan each sheet of organs using the small animal optical imaging system (**Figure 3C,D**).
    1. Use the same image acquisition parameters as described in step 2.4.
8. After scanning, place organs in 10% formaldehyde to enable subsequent processing for histology.

4. Quantification of Tumor Burden in the Fluorescence Images

1. In order to reduce the amount of auto-fluorescence in each multispectral scan, first unmix the files using software available with the small animal imaging system.
    1. Load first the *RFP: In Vivo* spectral file, followed by the *Autofluor: In Vivo Red* file in the Unmixed Images window and select **Unmix**.
2. Export the .bip files as 16 bit .tiff (unscaled) format .
3. Use **ImageJ** software (*ex vivo* organs and then the full mouse body with organs *in situ*).
    1. Use **File > Open** to open the 16 bit .tiff files of the organ scanning template files in ImageJ.
    2. Under **Image > Adjust > Brightness/Contrast**, choose to **Set** the **Minimum displayed value** to **0** and the **Maximum displayed value** to **+35353** and then under **Image > Lookup Tables**, select **Red Hot**. Note: Various animal models will require different brightness levels, but it is important to keep the brightness consistent throughout a given study.
    3. Using the **Freehand Selection** tool, draw a free-form region of interest selection (ROI) around each organ and use the **Measure** tool (CTRL+M) to calculate the surface area of each organ; record the surface **Area** of each organ in a spreadsheet.
    4. With the ROI drawn around each organ, right click within the ROI and select **Duplicate** to include only the organ.
    5. While looking at the individual organ, under **Image > Adjust > Threshold**, choose to **Set** the threshold of the image to a **Lower Threshold Level** of **+ 1200** and an **Upper Threshold Level** of **+ 1700** to select only the most brightly fluorescing regions and check to select **Dark Background**; select **OK**. Note: The images may require manual manipulation of the threshold sliders in ImageJ so that the previously brightest areas are selected for the analysis step, as shown above. Different disease models will require different threshold constraints, but it is important to keep the threshold levels consistent throughout a given study.
    6. Under **Analyze > Analyze Particles**, change the **Size (pixels ^ 2)** to **10-Infinity**, choose to **Show: Outlines**, check to select only **"Display results"** and click **OK** to measure both the areas and raw integrated densities of these bright regions, deemed tumors.
    7. Record the values of the surface **Area** and the **Raw Integrated Density** of each tumor selection displayed in the **Results** table in the same spreadsheet containing the organ surface areas.
    8. Under **Analyze > Tools > Calibration Bar**, add a calibration scale bar to the images of the organs. Note: In this example, this was done by changing the **Location** to **Upper Right**, the **Fill Color** to **Black,** the **Label Color** to **White**, the **Number of Labels** to **5**, the **Decimal Places** to **0**, the **Font Size** to **12** and the **Zoom Factor** to **4.0** and enabling **Bold Text**.
    9. Under **File > Save As**…, save the montage as a **.Tiff** and **.Jpeg**.
    10. Use **File > Open** to open the .jpeg file of the organs and then under **Insert > Text Box**, add organ labels by creating a text box below each of the three rows of organs.
    11. Use **File > Open** to open each of the 16 bit .tiff files of the full body scans in ImageJ in order of mouse number.
    12. Under **Image > Adjust > Brightness/Contrast**, choose to **Set** the **Minimum displayed value** to **0** and the **Maximum displayed value** to **+ 35353** and then under **Image > Lookup Tables**, select **Red Hot**.
    13. Under **File > Save As**…, save the montage as a .**Tiff** and **.Jpeg**.

5. Data Analysis

1. Add the tumor selection areas and raw integrated densities, respectively, for each organ of each mouse and record the total organ tumor area for each mouse.
2. Normalize the recorded area and raw integrated density values for the size of each organ by dividing the total tumor area and total raw integrated density values by the corresponding organ surface area (**Table 1**).
3. Calculate and plot the average of both the normalized tumor surface areas and the normalized raw integrated density values (**Figure 5A,B**).
4. Compare these average values to those obtained by visually counting the tumors that were visible to the naked eye during the inspection of each organ for metastatic lesions.

Representative Results

The metastatic mechanism of ovarian cancer is characterized by highly diffuse intra-peritoneal metastasis comprised of numerous lesions of varying size, including multiple small (< 2mm) lesions. Thus, use of RFP-labelled tumor cells (**Figure 1**) and optical imaging provides an alternative method to manual counting and measurement of lesion size. The development of tumor burden over time can be determined by weekly weighing of mice and measurement of abdominal girth to gauge potential presence of ascites. *In vivo* optical imaging provides a complementary approach to assess tumor burden (**Figure 2**). Ovarian cancer metastasizes to multiple sites in the peritoneal cavity and molecular drivers of site-specific metastasis are currently unknown. In addition to determination of overall tumor burden, following sacrifice careful dissection can be performed to evaluate and quantify site-specific patterns of metastasis. Thus, optical imaging of peritoneal organs *in situ* (**Figure 3A,B**) combined with *ex vivo* assessment of individual organs (**Figure 3C,D**) can provide useful data on overall vs organ-specific tumor burden. The scans presented in **Figure 3** represent the results of scanning the abdominal cavities and individual organs of mice with varying degree of tumor burden. For example, **Figure 3C** shows the most tumor burden in the omentum/pancreas, ovaries and mesentery of Mouse A. When compared to those same organs in Mouse B, a significant difference in signal is seen (**Figure 3D)**. The use of the organ scanning template (**Figure 4**) aids in identification of organ-specific tumor burden. The observation of differential tumor burden is confirmed quantitatively both in terms of tumor surface area and tumor signal intensity (**Table 1, Figure 5**).

The results shown in these figures and table were analyzed using a minimum and maximum display range of 0 to + 35353 so as to avoid any false-positive signals and auto fluorescence given off by the tissues. The varying degrees of signal seen in **Figure 2 and 3** illustrate the large range of tumor burden that can be imaged and quantified using this method. The data in **Table 1** illustrates this large range of applicability in quantifying both tumor surface area and signal intensity of the tumor. For example, when looking at the omentum/pancreas of Mouse A compared to that of Mouse B, the normalized tumor surface area is 26 times greater in Mouse A than Mouse B. Furthermore, the large range of signal intensity is also exemplified in the omentum/pancreas of Mice A and B; the normalized raw integrated density of Mouse A is 56 times greater than that of Mouse B. These results demonstrate the validity of comparing the tumor burden of several test subjects relative to each other, in terms of both tumor surface area and tumor signal intensity, using this quick method that does not require further histologic analysis.

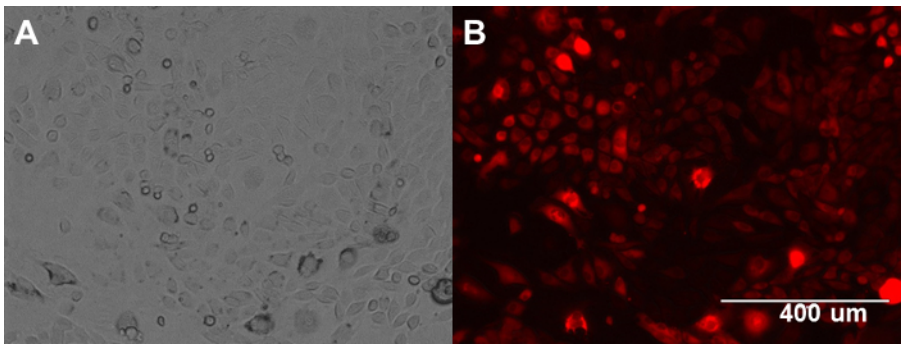

Figure 1. ID8 Murine Ovarian Cancer Cells Express RFP. (A) Phase image of ID8 cells. **(B)** Fluorescence image of ID8 cells. Please click here to view a larger version of this figure.

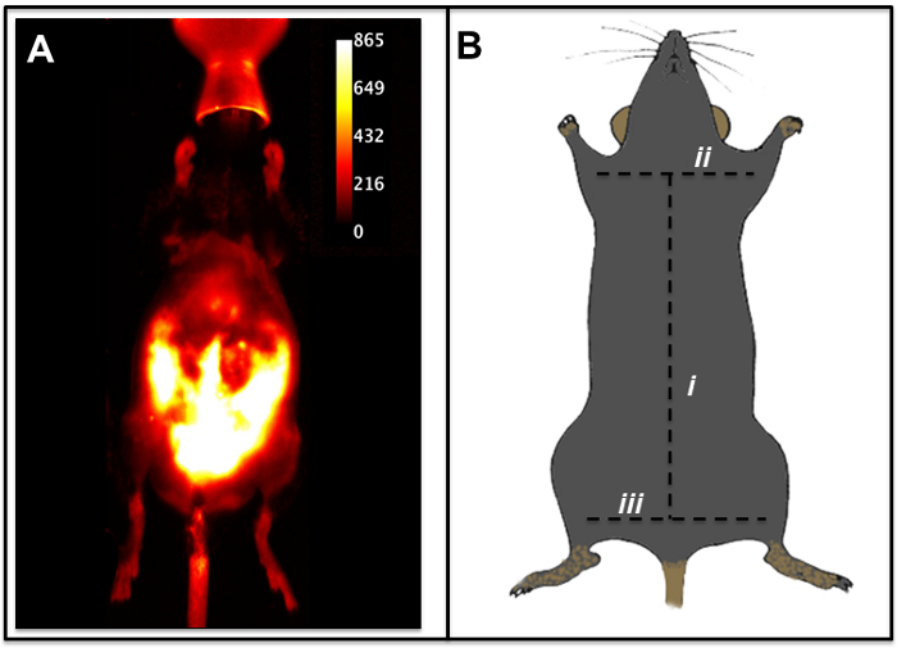

Figure 2. Live Optical Imaging of C56/Bl6 Mouse with Intra-peritoneal Tumor Burden. (A) Depilatory cream was used to remove hair from the ventral surface of the mouse prior to scanning. **(B)** Diagram showing initial ventral midline and lateral incisions. Please click here to view a larger version of this figure.

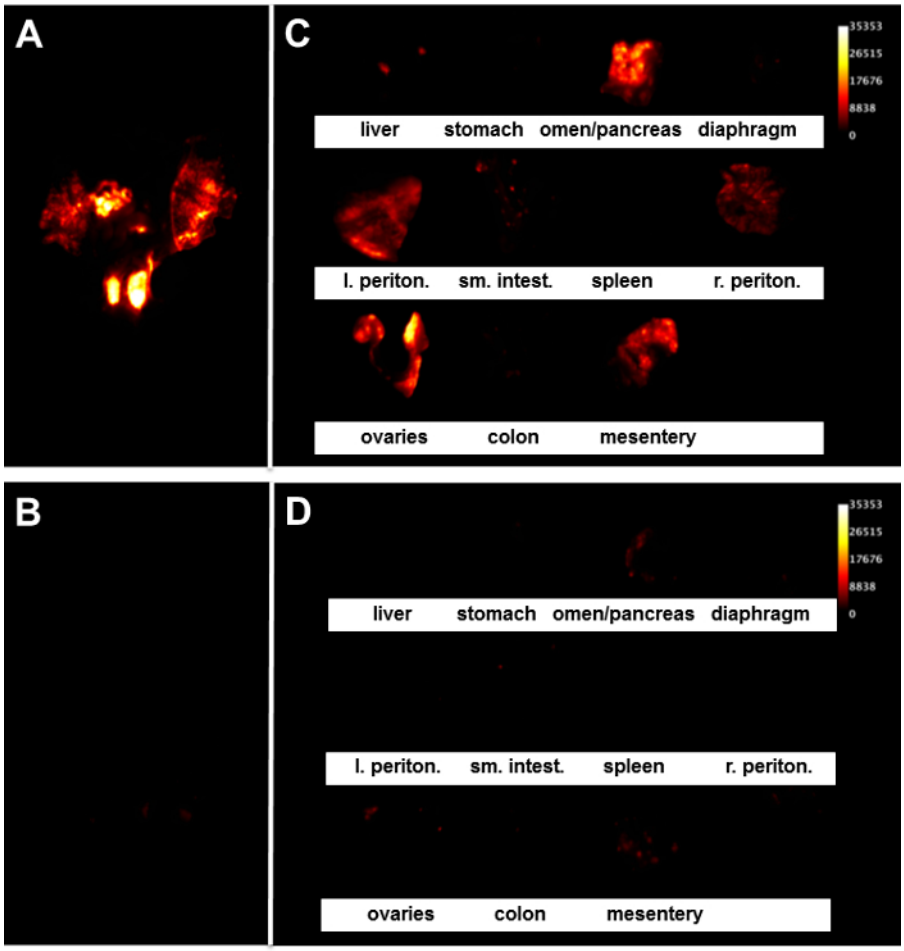

Figure 3. **Endpoint Scanning of Intra-Peritoneal Tumor Burden**. **(A, B)** Mice are first imaged with organs *in situ* (ventral side down) following opening of the ventral surface of the mouse. **(C,D)** Imaging of individual organs. Each organ is dissected, visual tumor burden recorded, and placed on the organ scanning template for analysis. Please click here to view a larger version of this figure.

MOUSE #: ____
DATE:

LIVER	STOMACH	OMENTUM/ PANCREAS	DIAPHRAGM
PERITONEUM LEFT	SMALL INTESTINE	SPLEEN	PERITONEUM INJ. SITE
OVARIES	LARGE INTESTINE/ COLON	MESENTERY	FAT PADS

Figure 4. Organ Scanning Template. Dissected organs are placed on the organ scanning template to maintain positional identification of individual organs during scanning.

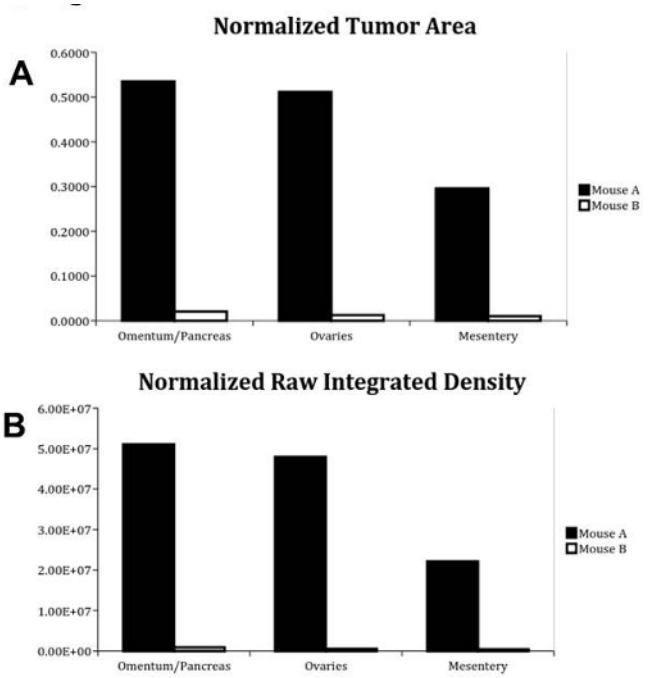

Figure 5. Normalized Quantitative Data for the Omentum/Pancreas, Ovaries and Mesentery. Data were analyzed as described in Protocol and as shown in **Table 1**. Mouse A refers to the mouse scanned in panel 3A; mouse B refers to the mouse scanned in panel 3B. **(A)** Normalized tumor area. Fluorescence signal quantification analysis of these three organs was conducted and quantified in terms of a ratio of tumor surface area to whole organ surface area for both mice. **(B)** Normalized raw integrated density. Fluorescence signal quantification analysis of these three organs was conducted and quantified in terms of a ratio of raw integrated density to whole organ surface area for both mice.

	Omentum/Pancreas	
	Normalized Tumor Area	**Normalized Raw Integrated Density**
Mouse A	0.5346	5.11E+07
Mouse B	0.0203	8.97E+05
	Ovaries	
	Normalized Tumor Area	**Normalized Raw Integrated Density**
Mouse A		4.79E+07
Mouse B	0.0123	5.62E+05
	Mesentery	
	Normalized Tumor Area	**Normalized Raw Integrated Density**
Mouse A	0.2951	2.22E+07
Mouse B	0.0098	4.15E+05

Table 1. Normalized Tumor Area and Normalized Raw Integrated Density values in the Omentum/Pancreas, Ovaries and Mesentery of both Mouse A and Mouse B. Post dissection and scanning, each organ was segmented and its fluorescence quantified to determine the proportion of tumor area to total organ surface area and the intensities of the fluorescence for these tumor areas. The omentum/pancreas, ovaries and mesentery were selected as they had the strongest fluorescence signals within both the positive and negative control groups.

Discussion

In contrast to studies using human ovarian cancer cells that must be conducted in immunocompromised mice, the protocol described above utilizes immunocompetent C57/Bl6 mice and syngeneic murine ovarian cancer cells. While this enables assessment of the potential role of immune infiltrates in tumor progression and metastasis, the presence of dark hair on the abdominal surface renders imaging less sensitive. Use of a depilatory to remove hair prior to imaging enhances image acquisition, but is time-consuming, particularly for experiments requiring longitudinal imaging. Hairless 'nude' mice can be used in this protocol and do not require depilatory-based hair removal. Furthermore, nude mice lack a functioning immune system, so can also be utilized to assess the growth of human ovarian cancer cells in experiments wherein the contribution of the immune system is not under analysis.

It should also be noted that the depth of intraperitoneal tumors also presents technical challenges, as optical fluorescence imaging will generally not detect tumors at depths greater than 5 mm. Furthermore, the prevalence of ascites fluid and the presence of ascitic tumor cells provide

additional complications. We have observed significant mouse-to-mouse variability in the formation of ascites fluid, even when injected with the same cell line. Thus in the presence of ascites, this method is preferable for end-point analysis of organ-specific tumor burden rather than routine longitudinal imaging of progression. In the current case, dramatic changes in tumor burden were noted between the example mice, such that cohorts of four to five mice will usually be sufficient for statistical analysis. In the case of less dramatic differences in tumor burden, additional experimental subjects will be needed to reach statistical power. While the examples shown herein differ significantly in tumor volume, optical fluorescence imaging can be used to detect much smaller differences in organ-specific tumor burden, particularly when compared to weight- or caliper-based measurements on small lesions (not shown).Resolution and sensitivity will vary with cell line and imaging system. In the present case, metastatic implants as small as 400 µm in diameter can be resolved and quantified. Alternative methods including contrast-enhanced computed tomography (CT), magnetic resonance (MR) imaging, and positron emission tomography (PET) have been described for longitudinal imaging[14,15]. Modalities including combined anatomic and functional imaging are also under development[16].

In regards to this proposed method for quantification of tumor burden, it should be noted that the determination of the fluorescence thresholds [Step 4.3.5.] is based on the intensity of the fluorescence seen in the organ images [Step 4.3.2.] and is chosen by the user to include only the most fluorescent regions relative to the rest of the organ. Varying threshold values were used in the analysis before determining the final threshold values used in this study. Once determined, these same thresholds were used for every organ analyzed so as to ensure consistency in the quantification of what is deemed metastatic tissue by the initial visual analysis done by the researcher. The determination of these thresholds is a critical step in the procedure and potentially could vary from one project to the other as determined by the individual researcher. In this case, a large lower threshold was used so as to capture only the highest intensity fluorescence signals. By using this same threshold for every organ analyzed, the analysis and results offer comparative quantitative results within the context of this cohort of mice. While this method is limited in quantification of absolute values of tumor surface area, it allows researchers the ability to compare relative normalized tumor burden among the same and different organs within cohorts of mice. It should be noted that this approach includes quantification of tumors that are not visible to the naked eye.

The unique metastatic mechanism of EOC results in widely disseminated intraperitoneal carcinomatosis involving multiple tissues and organs, rendering optimal cytoreductive surgery technically challenging. As complete cytoreduction positively correlates with overall survival, it follows that the development of new therapeutic strategies to combat intra-peritoneal metastasis is warranted. Assessment of therapeutic efficacy, however, is dependent on accurate quantitation of tumor burden, including evaluation of organ-specific metastasis. Using optical imaging of RFP-tagged tumor cells *in situ* corrected for organ autofluorescence, combined with careful *ex vivo* organ imaging, we have developed an approach to quantify intraperitoneal organ-specific tumor burden. Interestingly, our analyses show that preferred sites of metastasis in this model, as defined by tumor burden/surface area, are similar to those found in women with ovarian cancer (omentum, ovary, intestine, mesentery)[2,4,5]. Therefore this approach should have future utility in evaluation of experimental therapeutics designed to target metastatic disease.

Disclosures

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