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Magnetic resonance imaging assessment of carcinogen-induced murine bladder tumors

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Cover Letter

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November 2, 2018

Dear Dr. Myers,

I am pleased to to submit our revised manuscript entitled “Magnetic resonance imaging assessment of carcinogen-induced murine bladder tumors” for consideration for publication in *JOVE*. We feel that the manuscript has been strengthened by the careful review of editors and peers.

All research is new and not in consideration in another Journal. All conflicts of interest were disclosed and all authors contributed to the design and review of the manuscript.

We look forward to hearing from you.

Best regards,
Joshua J. Meeks, MD, PhD
Assistant Professor of Urology

TITLE:

Magnetic Resonance Imaging Assessment of Carcinogen-induced Murine Bladder Tumors

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KEYWORDS:

Urinary bladder neoplasms, magnetic resonance imaging, carcinogens, mice, BBN, bladder cancer

SUMMARY:

Murine bladder tumors are induced with the N-butyl-N-(4-hydroxybutyl) nitrosamine carcinogen (BBN). Bladder tumor generation is heterogeneous; therefore, an accurate assessment of tumor burden is needed before randomization to experimental treatment. Here we present a fast, reliable MRI protocol to assess tumor size and stage.

ABSTRACT:

Murine bladder tumor models are critical for the evaluation of new therapeutic options. Bladder tumors induced with the N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN) carcinogen are advantageous over cell line-based models because they closely replicate the genomic profiles of human tumors, and, unlike cell models and xenografts, they provide a good opportunity for the study of immunotherapies. However, bladder tumor generation is heterogeneous; therefore, an accurate assessment of tumor burden is needed before randomization to experimental treatment. Described here is a BBN mouse model and protocol to evaluate bladder cancer tumor burden *in vivo* using a fast and reliable magnetic resonance (MR) sequence (true FISP). This method is simple and reliable because, unlike ultrasound, MR is operator-independent and allows for the straightforward post-acquisition image processing and review. Using axial images of the bladder, analysis of regions of interest along the bladder wall and tumor allow for the

calculation of bladder wall and tumor area. This measurement correlates with *ex vivo* bladder weight (r_s= 0.37, p = 0.009) and tumor stage (p = 0.0003). In conclusion, BBN generates heterogeneous tumors that are ideal for evaluation of immunotherapies, and MRI can quickly and reliably assess tumor burden prior to randomization to experimental treatment arms.

INTRODUCTION:

Bladder cancer is the fifth most common cancer overall, responsible for approximately 80,000 new cases and 16,000 deaths in the United States in 2017[1]. After about 30 years without significant advances in the systemic treatment of bladder cancer[2], recent anti-PD-1 and anti-PD-L1 checkpoint inhibitor trials have demonstrated exciting and occasionally durable responses in patients with advanced urothelial carcinoma[3-5]. However, only approximately 20% of patients show an objective response to these treatments, and further studies are needed to expand the effective use of immunotherapy in patients with bladder cancer.

Murine bladder cancer models are critical tools in preclinical evaluation of novel treatments[6,7]. In order to control for tumor size when randomizing mice to different treatments, tumor burden must be assessed and controlled between treatment groups. Previous studies have used ultrasound or bioluminescence to evaluate orthotopic cell line-based bladder cancer models[8-11]. However, both techniques present several disadvantages. Ultrasound measurements can be influenced by skills of the operator and lack three-dimensional features and high spatial resolution. Bioluminescence methods can only provide semi-quantitative evaluation of the tumor cells and do not allow for visualization of bladder anatomy and morphology. Furthermore, bioluminescence can only be used with cell line-based models, which express bioluminescent genes in hairless mice or mice with white coats.

Magnetic resonance imaging (MRI), on the other hand, offers unique flexibility in the acquisition of high-resolution anatomical images, exhibiting a broad range of tissue contrast that enables accurate visualization and quantitative assessment of tumor burden without the need to express bioluminescent properties. MR images are more easily reproducible with the appropriate analysis pipelines and guaranteed 3-D visualization of the bladder. The biggest limitations of MRI are the length of time necessary for an examination and associated high costs that limit high throughput assays. However, several studies have shown that MR sequences can provide high-quality diagnostic images that can be used to effectively detect and monitor cell line-based bladder tumors; thus, they may be used for high throughput analysis[9,12].

Here, we describe a non-invasive MR-based method to reliably and efficiently characterize carcinogen-induced bladder tumors in mice. To accomplish this, we use a fast imaging with steady state precession MR technique (true FISP), which guarantees short scanning sessions while still providing high quality and high spatial resolution (~100 microns) for the detection and measurement of bladder tumors[13]. Furthermore, to confirm the accuracy of this non-invasive MRI assay, we describe the correlation between MRI-derived parameters and *ex vivo* bladder weight as well as pathologically-confirmed tumor stage.

PROTOCOL:

All methods described here have been approved by the Institutional Animal Care and Use Committee (IACUC) of Northwestern University.

1. Induction of tumors with BBN

1.1 Obtain male C57/BL6 mice, each at least 6 weeks old.

NOTE: Male mice develop bladder cancer more quickly and consistently than female mice[14,15].

1.2 Add N-nitrosobutyl(4-hydroxybutyl) amine (BBN) at a dose of 0.05% to the drinking water. Store it in an opaque container and provide it *ad libitum* as drinking water to mice[16].

NOTE: Storing the BBN solution in a clear container will degrade the carcinogen[17].

1.3 Change the 0.05% BBN water twice per week.

1.4 Monitor the animals by inspecting for signs of distress associated with bladder tumors including hematuria, firm bladder, and masses. Inspect the mice twice per week or in accordance with local IACUC guidelines.

1.5 Expect the tumors to develop between 16 and 24 weeks of exposure[18].

2. MRI setup

2.1 Perform a subcutaneous injection of sterile saline (0.1–0.2 mL using a 25–27 G needle and 1 mL syringe) 10 min prior to MRI to facilitate bladder filling.

2.2 Anesthetize each mouse with a gas mixture of 100% O_2 and isoflurane (2%–4% as necessary). Verify an adequate plane of anesthesia by testing the withdrawal reflex (toe pinch) before proceeding.

2.3 Transfer the mouse to the imaging holder outfitted with a nosecone for delivery of inhaled isoflurane (0.5%–3%).

2.4 Monitor body temperature and respiration using a rectal temperature probe connected to the physiological recording computer.

NOTE: Normal body temperature (36–37 °C) is maintained using the recirculating hot water circuit built into the animal MR holder. Temperature is measured through a rectal sensor and recorded on the physiological monitoring computer using dedicated physiological monitoring software. The same system is used to record the respiration and electrocardiogram signals measured through a pneumatic pillow placed under the rib cage and *via* 3-lead electrocardiogram electrodes. The respiration signal is also used for triggering MRI acquisition and reducing artifacts associated with respiration motion.

3. MRI image acquisition

3.1 Utilize a quadrature body coil for excitation.

3.2 Place a 4-channel receiver coil on the lower abdomen of the mouse being scanned to enable optimized detection of signals from the region of interest.

3.3 Initiate automatic adjustments through the integrated imaging software to acquire a tri-axial set of images of the whole mouse body. From this reference set of images, identify the region of interest (in this case, the bladder region).

3.4 Acquire three sets of orthogonal-sliced images along the axial, coronal, and sagittal planes using radiological frames of reference.

3.5 Utilize the true FISP imaging sequence (included as one of the features in the integrated imaging software) with the following MR parameters: TR = 900 msec, TE = 2 ms, FA = 70, 14 averages.

NOTE: This set of parameters allows for rapid imaging with high diagnostic quality, including T1/T2 weighting in <10 min per mouse.

3.6 Spatial resolution and slice thickness are determined by geometric parameters selected by the user through the graphical interface of the integrated imaging platform. This results in a series of slices across the whole bladder of 0.5 mm thickness with an in-plane resolution of 0.148 mm.

4. MR image analysis

4.1. Identify the set of slices of 0.5 mm thickness and in-plane resolution of 0.148 mm covering the whole bladder.

4.2. Export to the medical image analysis software by selecting the folder with corresponding images in ANALYZE format.

4.3 Select “representative axial view” at the center of the bladder for quantitative analysis by scrolling through the generated images and identifying a slice at the midpoint of the bladder, which allows for visualization of the bladder wall and lumen.

NOTE: The center slice should be the chosen one with the largest diameter.

4.4 Carefully delineate the region of interest (ROI) by manually tracing the boundaries around the outer edge of the bladder (BLA_{out}) and around the inner lumen (BLA_{in}) of the bladder (see schematic and representative figures in **Figure 2**) in the selected representative

axial view.

4.5 Subtract the inner lumen from the outer edge to calculate the surface area of the bladder wall.

$$BLA_{wall} = BLA_{out} - BLA_{in}$$

NOTE: The surface area of a control bladder with no tumor is expected to be less than that with a bladder tumor.

5. Euthanasia and dissection of bladder

5.1 After 20 weeks of BBN exposure, euthanize the mice using standard operating procedures in accordance with local IACUC guidelines.

5.2 Clean the area of incision with 70% ethanol, then grasp and lift the abdominal wall skin with forceps.

5.3 Make a midline incision from the pubic symphysis to the xiphoid process.

5.4 Sharply incise the peritoneal cavity by grasping with forceps and incising with scissors.

5.5 Identify the bladder, which is located in the midline lower abdomen.

5.6 Identify and cut the median umbilical ligament connecting the dome of the bladder to the umbilicus and abdominal wall.

5.7 Grasp the dome of the bladder with forceps to provide countertraction and dissect the bladder away from surrounding structures, including the seminal vesicles, rectum, and fat.

5.8 Identify the ureters entering the bladder and cut with scissors close to the bladder.

5.9 Lifting the bladder cephalad, cut the urethra with scissors and remove the bladder.

5.10 Immediately weigh the bladder after rinsing it with PBS.

6. Histologic examination of bladder tissue

6.1 Fix the bladder tissue in 10% neutral buffered formalin for 36–48 h at room temperature (RT).

6.2 Embed the tissue in paraffin blocks, cut the slides for subsequent examination, and stain the slides with hematoxylin and eosin for microscopic examination as described previously[19,20].

6.3 Perform a microscopic examination of the mouse bladder at low (2.5x and 10x) and high

(20x and 40x) magnifications, examining for macroscopic lesions, hyperplasia, carcinoma *in situ*, papillomas, papillary tumors, and invasive neoplasms[19,21].

REPRESENTATIVE RESULTS:

Using the protocol described (**Figure 1**), bladder tumors were induced in C57/B6 male mice. MRI was performed at 16 weeks, and mice were euthanized at 20 weeks. *Ex vivo* bladder weights (BW) for each mouse were recorded. Slides were stained with hematoxylin and eosin, and all histology slides were reviewed for tumor stage.

To analyze the tumor burden using MR, the bladder wall inner lumen (BLA_{in}) was subtracted from the bladder wall outer lumen (BLA_{out}) to calculate the thickness of the bladder wall (BLA_{wall}) (**Figure 2**). Representative true FISP MR images, bladder wall 3-D reconstructions, and pathologic images of a control mouse (i.e., no tumor) are shown in **Figure 3A–F,** and a mouse with a large tumor is shown in **Figure 3G–L**.

The MRI-derived parameter BLA_{wall} correlates weakly with *ex vivo* BW ($r_s = 0.37$, $p = 0.009$; **Figure 4**). Examination of the MRI-derived BLA_{wall} parameter and BW data demonstrates an association with tumor stage (Kruskal-Wallis test MRI $p = 0.0003$, **Figure 5A**; BW $p = 0.0006$; **Figure 5B**), as well as an association when stratifying pathology by non-muscle-invasive bladder cancer and muscle-invasive bladder cancer (Mann-Whitney U test MRI $p = 0.0002$, **Figure 5C**; BW $p < 0.0001$, **Figure 5D**). The performance of BLA_{wall} and BW to determine muscle-invasive bladder cancer is shown in **Figure 5E**. The area under the curve (AUC) for BLA_{wall} (AUC = 0.81, 95% CI 0.68–093) is statistically similar to AUC for BW (AUC = 0.89, 95% CI 0.80–0.98; $p = 0.30$).

FIGURE AND TABLE LEGENDS:

Figure 1: Schema for bladder tumor induction with BBN and timing of MRI and euthanasia. BBN is administered *ad libitum* at a concentration of 0.05% in drinking water. Mice undergo MRI at 16 weeks. Mice are euthanized at 20 weeks and bladders of each are examined with immunohistochemistry.

Figure 2: Schematic graphical depiction of method to obtain BLA_{wall} and representative MR image with corresponding outlines. Using intensity of MRI images, the outer wall of a bladder was identified and an outline was drawn in red (BLA_{out}). The hyperintense bladder lumen was outlined in green (BLA_{in}), and the corresponding bladder lumen area was obtained. Subtraction of these two quantities yielded the BLA_{wall} parameter, which corresponds to the light gray disk in the graphical image.

Figure 3: Representative true FISP MR images, bladder wall 3-D reconstructions, and pathologic images of a control mouse (i.e., no tumor) (A–F) and a mouse with a large tumor (G–L). **(A)** Representative MR image of a mouse with no tumor. **(B)** Segmentation of bladder wall area (BLA_{wall}), outlined in red, defined as the area between the bladder lumen (BLA_{in}) and outer bladder wall (BLA_{out}). **(C)** 3-D rendering of the bladder wall from a control mouse, generated by defining BLA_{wall} at every slice through the bladder. Green arrows illustrate the

bladder on a 2-D image translated to 3-D rendering. **(D)** 3-D rendering of a cut-out of BLA_{wall} from a control mouse. **(E)** Low power (2.5x) and **(F)** high power (10x) images of the same mouse bladder. **(G)** Representative MR image of a mouse with a large tumor. **(H)** Segmentation of bladder wall area (BLA_{wall}), outlined in red, defined as the area between the bladder lumen (BLA_{in}) and outer bladder wall (BLA_{out}). **(I)** 3-D rendering of the bladder wall of a mouse with a large tumor. **(J)** 3-D rendering of a cut-out of the bladder of a mouse with a large tumor, generated by defining BLA_{wall} at every slice through the bladder. Green arrows illustrate the bladder on a 2-D image translated to 3-D rendering. **(K)** Low power (2.5x) and **(L)** high power (10x) images of the same mouse bladder.

Figure 4: Spearman correlation between the MRI-derived BLA_{wall} and final bladder weight.

Figure 5: Comparisons of pathologic stage and MRI-derived parameter BLA_{wall} in 47 mice. (A) Comparison of all pathologic stages and MRI BLA_{wall} (Kruskal-Wallis test). **(B)** Comparison of all pathologic stages and bladder weight (Kruskal-Wallis test). **(C)** Comparison of non-muscle-invasive bladder cancer (stage ≤T1) and muscle-invasive bladder cancer (stage ≥T2) with MRI BLA_{wall} (Mann-Whitney U test). **(D)** Comparison of non-muscle-invasive bladder cancer (stage ≤T1) and muscle-invasive bladder cancer (stage ≥T2) with bladder weight (Mann-Whitney U test). **(E)** ROC curve of the MRI-derived bladder area and final bladder weight in determining muscle-invasive bladder cancer (stage ≥T2). The listed p-value is the difference between the two AUCs.

DISCUSSION:

Accurate imaging of tumor models is necessary for appropriate pre-euthanasia staging and animal randomization prior to initiation of experimental treatment. Using the procedure presented here, we demonstrate methodology to (1) generate bladder tumors using the BBN carcinogen and (2) stratify bladder tumor burden through the use of MR. An MR-derived area measurement (BLA_{wall}) correlates significantly with *ex vivo* bladder weight and is associated with pathologic tumor stage.

By adopting a rapid imaging approach with short acquisition times at high spatial resolution (true FISP) and high diagnostic quality, we can conduct high throughput assays of mice at intermediate stages of tumor development, prior to treatment randomization. Our report is consistent with prior reports of MR imaging of cell line-based tumor implants[9,12] and confirms its potential as a tool to optimize large subject number drug studies.

In this MRI protocol, it is critical to image the mouse with a full bladder to obtain high quality images and delineate the differences between the tumor and bladder lumen. We find that injecting each mouse with saline 10 minutes before imaging allows for adequate imaging of the bladder. Further critical steps include reliable triggering of MRI acquisition using the respiration signal detected with a pneumatic pillow placed under the mouse rib cage and acquisition of an adequate number of MR slices that enables coverage of the whole bladder.

Other options for imaging development and progression of murine bladder tumors include

ultrasound[8] and bioluminescence[10,11]. Micro-ultrasound imaging of implanted MBT-2 cells detected tumors in 15 mice, 13 of which were histologically confirmed to have tumors[8]. Ultrasound volume correlated significantly with stereoscopic volume of tumor, but tumor weight and stage were not investigated[8]. Bioluminescence has been used to accurately monitor cell line-based tumor implants, but it cannot be used to monitor carcinogen-induced cancers without transplanting carcinogen-derived tumors from one mouse to another. The ability to accurately monitor carcinogen-induced cancers is critical, as these models have several advantages over cell line models. Cell line-based models are genetically homogenous and derived from tumors that have already evaded immunosurveillance, and implanted tumors grow rapidly without a chronic inflammatory microenvironment[22]. The BBN model has been used successfully for over 30 years, and it remains a critical model for the understanding of bladder cancer development and treatment[23-25]. Furthermore, the BBN model demonstrates mutational and gene expression profiles similar to human bladder cancer, while still retaining the intact immune system to allow for the study of potential immunotherapeutic agents[26,27].

Availability of dedicated small animal MRIs as shared resources at multiple institutions makes this techniques advantageous and practical for basic research and screening of novel therapies. However, there are some limitations. Mice were imaged only at one timepoint, not continuously during the development of tumors. However, based on our statistical results, we suggest that the single timepoint value is able to accurately stratify mice into groups by tumor size and stage, and it represents an ideal, non-invasive parameter to classify and assign subjects to different groups. Multiple tumor stages were generated using BBN, ranging from Ta to T4. However, these may be stratified (as suggested in **Figure 5C-D**) as muscle-invasive (T2 or greater) and non-muscle invasive (T1 or less), as this is standard management in human bladder cancer[28].

Another potential limitation is that the BLA_{wall} parameter was derived using a single slice through each bladder and not all available slices covering it. These criteria were chosen to reduce analysis pipeline requirements (i.e., requirement of drawing multiple ROIs across multiple slices) and were deemed sufficient for a fast, quantitative assay. More complex volumetric analysis can be conducted on the subjects (i.e., shown for illustrative purposes in **Figure 3**) but would inevitably require more effort and costs. Automated image processing algorithms can be used for automatic delineation of bladder region; however, these methods suffer from intrinsic variability of bladder shape and size among individual mice and require significant testing and validation prior to reliable adoption in a preclinical study[29].

Qualitative assessment of volumetric data suggest that this single slice method is sufficient for this type of assay. However, it is possible that more advanced assays may require this additional data/image processing step. From the acquisition point of view, there are several additional scans that could be acquired, which may further increase the ability to predict progression of tumors while also revealing more subtle tumor microenvironment changes. These additional techniques include dynamic contrast enhanced MRI, diffusion weighted MRI, and other sequences[30] that enable a comprehensive, multi-parametric characterization of the bladder wall. However, consideration of cost and efficiency led us to confine our assay to the one

described in this protocol.

In conclusion, we describe the methodology for T1/T2-weighted rapid imaging MR sequences (true FISP) to acquire multi-slice images covering the entire mouse bladder. We demonstrate that these images can be used to determine the extent of tumor in a carcinogen-based model of murine bladder cancer. MRI data correlates with bladder tissue weights and is associated with tumor stage. These results support the use of this fast and reliable MRI assay to stratify mice prior to experimental treatment randomization.

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DISCLOSURES:

The authors have nothing to disclose.

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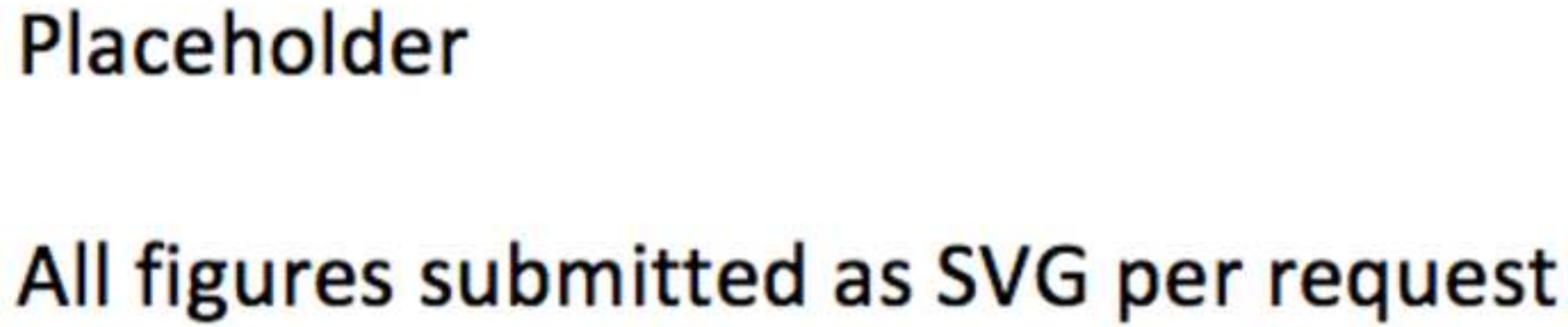

Figure 1

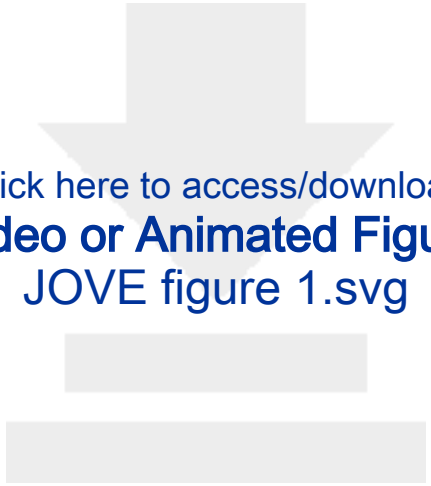
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Figure 3

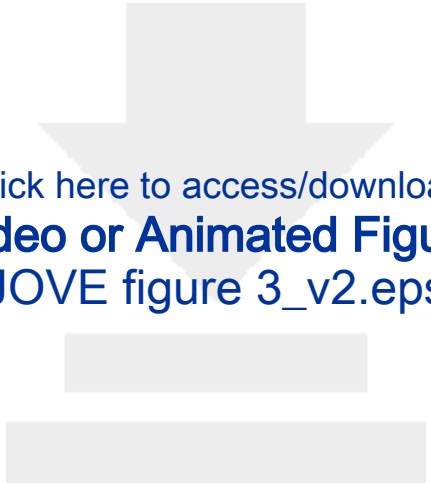
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Figure 4


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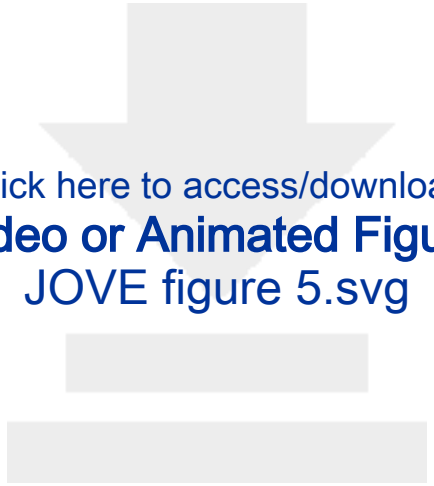
Figure 5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Name of Material/ Equipment	Company
C57BL/6 mice	The Jackson Laboratory
N-butyl-N-(4-hydroxybutyl)nitrosamine carcinogen (BBN)	TCI American
0.9% normal saline	Hospira, Inc
Isoflurane	Piramal HealthCare
7Tesla ClinScan MRI	Bruker
Syngo	Siemens
Model 1030 Monitoring & Gating System	Small Animal Instruments, Inc. (SAII)
Formalin, Neutral Buffered, 10%	Sigma
Eosin Y	Fisher Scientific
Hematoxylin	Fisher Scientific
Jim7	Xinapse Systems
GraphPad Prism v7.04	Graphpad
R v3.4.2	The R Project for Statistical Computing
R package pROC v1.10.0.	The R Project for Statistical Computing

Catalog Number	Comments/Description
664	Mice
B0938	Carcinogen
NDC 0409-488-02	
60307-120-25	Anesthetic
NA	Dedicated Small Animal Imaging MRI
NA	MR Integrated Imaging Software
NA	Small animal physiologic monitoring
HT501128	Fixative
NC1093844	Histologic staining agent
23-245651	Histologic staining agent
NA	Medical image analysis software
NA	Graphing software
NA	Statistical software
NA	ROC analysis

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Author(s):	Alexander P. Glaser MD, Daniele Procissi PhD, Yanni Yu, Joshua J. Meeks MD PhD

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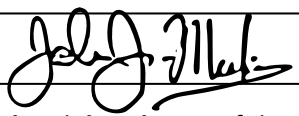
Signature:	
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Editorial comments:
Changes to be made by the author(s) regarding the manuscript:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Thank you. Minor grammatical changes have been made throughout the manuscript.

2. Please provide an email address for each author.

This has been provided in the title page.

3. Keywords: Please provide at least 6 keywords or phrases.

An additional keyword was provided to total 6. This is located on the title page.

4. Please remove commercial language and replace it with generic terms: Jim7, Xinapse Systems Ltd, etc.

Thank you. This language has been removed.

5. 1.5: How to confirm that tumors have been developed?

The only way to confirm that tumors have been developed is an imaging study as we describe in this manuscript.

6. 2.1: Please mention how proper anesthetization is confirmed.

This has been added to methods section 2.2.

7. 5.2: How to remove bladder tissue?

Additional details have been added to section 5.

For how long and at what temperature is the tissue fixed in 10% formalin? How histologic examination is done? What are the staining agents? Please add more details here or provide relevant references.

Additional details have been added, now listed as section 6, as requested.

8. Figure 3: Please explain what the green arrows and red circle represent in the figure legend.

Additional detail has been added to the Figure 3 legend.

9. Discussion: Please discuss critical steps within the protocol.

A paragraph describing critical steps has now been added to the discussion.

10. References: Please do not abbreviate journal titles.

References were previously cited using the JoVE EndNote Styling, which does abbreviate journal titles. The style in this manuscript has now been changed to include full journal names as requested.

11. Please revise the table of the essential supplies, reagents, and equipment to include the name, company, and catalog number of all relevant materials.

A revised materials table has been provided.

Reviewers' comments:
Reviewer #1:

Manuscript Summary:
This is a straightforward manuscript describing MRI to assess tumour burden in a mouse model of carcinogen-induced bladder cancer. The authors present the benefits and limitations of the model well and highlight how the technique may address a major challenge in the field - that BBN-induced tumours are highly heterogeneous. Indeed, the ability to stratify tumours according to stage prior testing experimental therapy should lead to greater consistency in reported results. While the manuscript is well written and organised, there are a few minor concerns, detailed below.

Major Concerns:
No major concerns

Minor Concerns:
1. MRI or MR is never defined in the text - it would be convention to define the acronym at first use.

MRI is now defined in the introduction. Thank you.

2. In the paragraph describing limitations (lines 67-75), it may be appropriate to discuss availability of MRI for mice. Is this technique/are the machines widely available, for example?

Thank you for bringing this up. Dedicated small animal MRIs are becoming more common and we have added a sentence regarding use of MRIs as shared resources into the discussion.

3. In the Protocol:
1.1 It is indicated that male mice should be used for this protocol, but no explanation for why is provided. This should be mentioned or sex should not be specified.

Thank you for this comment. Male mice develop bladder cancer more quickly than female mice in the BBN model, and a fair amount of work has been done to investigate why this is, so this should be emphasized to the reader. Additional details and citations have been added to the manuscript.

2.1 and 2.2 should likely be inverted? Might it make more sense to inject animals with water prior to anesthetising them?

Yes, thank you, this has been corrected.

2.4 Monitor body temperature and respiration is mentioned, but no indication of how is mentioned - by instrumentation, by eye?

Thank you for mentioning this critical point. Monitoring is performed with instrumentation. Additional details have been added to the methods, discussion, and table of materials.

5.1 As euthanasia protocols can vary widely by institute, it would be better to remove the phrase "of CO2 euthanasia followed by secondary cervical dislocation" (line 135) leaving only "using standard operating procedure in accordance with local IACUC guidelines.

Thank you, this statement has been adjusted in accordance with your recommendation.

4. In the representative results, the importance of correlation between bladder weight and bladder wall measurements is over-emphasised with respect to the correlation coefficient. An rs of 0.37 is not a strong correlation, as the strength of correlation decreases as the correlation coefficient approaches 0. The p-value only indicates whether the relationship measured occurred by chance. Thus, this is a relatively weak correlation (rs = 0.37), however, it is likely not due to chance (p=0.009). Additionally, it might be helpful to show the best fit line, as there appears to be one outlier in the graph with a much thinner bladder wall, and it is unclear how much this point drives the relationship.

Thank you for your careful review. We agree that rs of 0.37 is not a strong correlation, and have changed the wording in the text to reflect this. Removing the outlier as you mention increases the spearman coefficient. We opted not to place a fit line through the graph, as bets fit (i.e., regression) lines are not typically used for correlations.

5. In figure 5, p-values are presented, however, the tests used to generate them are not stated. The test used and the variables measured should be stated in the legend. Appropriate tests for the data in 5A and 5B would include a nonparametric Kruskal-Wallis test, with a post-test to correct for multiple comparisons, in which the authors compared all tumour stages to the control T0 stage. In 5C and 5D, a nonparametric Mann-Whitney would be an appropriate test to determine statistical significance.

Thank you. Indeed the nonparametric Kruskal-Wallis and Mann-Whitney tests were used as you describe. This is now included in the figure legend as suggested.

6. It is stated that "…the MRI derived BLAwall parameter and the BW data demonstrates a direct association with tumour stage…" (line 153-154). This is also likely overstated as the bladder wall values overlap almost entirely in Ta, T1, T2, and T4 tumours (5A). The same can be said for weight and all tumour stages except T3, T4 (5B). Analysis by Kruskal-Wallis testing will reveal statistically significant differences among the different groups. Those differences should be emphasised. Importantly - the potential lack of a statistically significant difference across all tumour stages does not diminish the potential applicability of the technique, however, the limitations of the "fast quantitation" analysis should be clear.

The word “direct” has been removed from the manuscript as we agree that this may be overstating the association. In addition, as you suggest, the name of the statistical tests performed was added.

7. In the discussion, it is emphasised that this technique will enable investigators to stratify animals prior to experimentation. It would be of interest if the authors commented on the very diverse range of

tumours analysed in the course of this study. Tumours ranging in stage from Ta to T4 are reported - were all of these stages observed after 20 weeks of BBN treatment? How might one stratify such diversity?

We have added an additional statement and citation regarding tumor stratification into muscle-invasive (T2 or greater) and non-muscle invasive (T1 or less), as is standard management in human bladder cancer.

Reviewer #2:

Manuscript Summary:
The manuscript by Glaser et al., descripts a detailed MRI protocol to evaluate tumor burden in situ, particularly the BBN-induced bladder cancer (BCa). Like human, male mice are much more susceptible than females to the BBN-induced BCa (Kaneko and Li, 2018). A recent report from this group also demonstrated that the BBN carcinogen induced BCa recapitulates molecular features of human muscle invasive BCa (Fantini et al., 2018). However, there is a lack of effective tools to accurately monitor bladder tumor burden longitudinally. A reliable MRI based protocol to assess tumor size and stage is timely and invaluable for the field of BCa study. Therefore, this manuscript is suitable for publication. A few minor comments:

Minor Concerns:

1. A better description of MR image analysis is recommended. Specifically, how the representative axial view is selected (4.3), and how the ROI is determined to draw BLAout and BLAin.

Thank you for your review and comments. Additional details have been added to this section as suggested.

2. Figure 4 and 5, please indicate the number of mice used for the analysis, and the statistic method used in Figure 5A and B. Please also highlight in Figure 5A and B which two groups are compared.

Additional details have been added regarding the statistical tests used for Figures 5A-B (Kruskal-Wallis test), and for Figures 5C-D (Mann-Whitney tests). The number of mice is also now included.

3. Recent advances of using the BBN model should be referenced as well.

Thank you. Additional citations, including Kaneko and Li 2018 have been added to the discussion as you suggest.

Editorial comments:

1. The editor has formatted the manuscript to match the journal's style. Please retain the same.

This has been done. Thank you.

2. Please address all the specific comments marked in the manuscript.

This has been done in the submitted R2 manuscript. Thank you.

3. Once done please highlight 2.75 pages of the protocol including heading and spacing. This should be the most cohesive story and will be used for filming.

Done. Sections 1-5 are highlighted as the histologic examination can vary depending on the purpose of the investigator and is listed here for the representative results.

4. Please upload new figure 3 as this is not opening.

Done (submitted as a .eps file now), sorry for the problem with the prior figure.

Thank you for your careful review of our manuscript.